REMARKS

Claims 1-38 are pending. Claims 13-27 and 29-38 have been withdrawn. Herein, Applicants have amended pending claims 1-5, 10-11, and 28, cancelled claims 6-9 and 12 and added new claims 39-43. Accordingly, claims 1-5, 10-11, 28 and 39-43 are being examined.

Support for amended claims 1-5, 10-11 and 28 and new claims 39-43 may be found in the claims and specification as originally filed. Accordingly, these changes do not involve new matter and Applicants respectfully request entry of these changes.

Support for amended claim 1 may be found in the specification as originally filed at page 16, paragraph [0048] last sentence, Table 2 at page 8, and originally filed claims 4, 6 and 7.

Support for amended claims 2-5, 10-11 and new claim 41 may be found in the specification as originally filed at page 1, paragraph [0003]; page 2, paragraph [0005], originally filed claim 8.

Support for amended claim 28 may be found in the specification as originally filed at page 15, paragraph [0045].

Support for new claims 39 and 40 may be found in the specification as originally filed at page 16, paragraph [0048].

Support for new claim 42 may be found in the specification as originally filed at pages 14-15, paragraph [0044].

Support for new claim 43 may be found in the specification as originally filed at pages 14-16, paragraphs [0044-0048].

In accordance with the changes to the claims and the remarks that follow, Applicants respectfully request reconsideration of the outstanding rejections.

ITEM 1: RESTRICTION REQUIREMENT

At page 2 of the outstanding Office Action, the Office has made the Restriction requirement final. Accordingly, claims 1-38 remain pending, claims 13-27 and 29-38 are withdrawn from consideration as being directed to non-elected subject matter and claims 1-12 and 28 are being examined. No response is due.

ITEM 2: PRIORITY

The Office acknowledges Applicants' claim for priority to provisional application 60/249,762, filed November 17, 2000. No response is due.

ITEM 3: INFORMATION DISCLOSURE STATEMENT

At pages 2-3 of the outstanding Office Action, the Office has indicated that reference Arima et al., ("Effects of Extracellular Matrix on Rat Kupffer Cell Functions in Vitro" 1999, Cells of the Hepatic Sinusoid, 7:68:69) was not found in the application file nor was it found upon a search by the Examiner.

Applicants have thus far not been able to obtain the reference but Applicants will continue searching for the correct cite and will provide it to the Examiner upon retrieving it.

ITEM 4: REJECTION UNDER 35 U.S.C §102(b)

At pages 3-7 of the outstanding Office Action, the Office rejected the following under 35 U.S.C. §102(b):

(i) claims 1-8, 10-12 and 28 as allegedly anticipated by Pulford et al., (Clin Exp Immunol. 1980) (hereafter Pulford),

(ii) claims 1-12 and 28 as allegedly anticipated by Gendrault et al., (Pathobiology, 1991) (hereafter Gendrault), and

(iii) claims 1-8, 10-12 and 28 as allegedly anticipated by Yoshioka et al., (Veterinary Immunology and Immunopathology, 1997) (hereafter Yoshioka).

Applicants respectfully disagree.

Applicants' invention is directed to a composition comprising isolated human replicating Kupffer cells, wherein the replicating Kupffer cells express CD68, do not express TGFβ, and at least some of the Kupffer cells have undergone cell division during culture *in vitro,* but wherein the composition does not comprise other Kupffer cells that do not express CD68 and that express TGFβ. Further, in one embodiment, Applicants use limiting dilution methodology to isolate a clonal population of replicating Kupffer cells.

THE LEGAL STANDARD FOR NOVELTY

To anticipate a claim, a prior art reference must disclose every limitation of the claimed invention, either expressly or inherently. Atlas Powder Co. v. E.I. du Pont de Nemours & Co., 750 F.2d 1569, 1574, 224 USPQ 409, 411 (Fed. Cir. 1984). Each and every element of the claimed invention must be disclosed in a single prior art reference in a manner sufficient to enable one skilled in the art to reduce the invention to practice, thus placing the invention in possession of the public. W.L. Gore & Assocs., Inc. v. Garlock, Inc., 220 USPQ 303 (Fed. Cir. 1983), *cert. denied* 469 U.S. 851, 105 S.Ct. 172 (1984); Scripps

Clinic & Research Found. v. Genentech, Inc., 927 F.2d 1565, 1576-7 (Fed. Cir. 1991), *clarified, on recons.*, 1991 U.S.App. LEXIS 33,486 (Fed. Cir. 1991). The absence of even a single element from a prior art reference negates anticipation. Atlas Powder Co. v. E. I. Du Pont de Nemours & Co., 750 F.2d 1569, 1574 (Fed. Cir. 1984).

To serve as an anticipation when the reference is silent about the asserted inherent characteristic, such gap in the reference may be filled with recourse to extrinsic evidence. Such evidence must make clear that the missing descriptive matter is necessarily present in the thing described in the reference, and that it would be so recognized by persons of ordinary skill. Continental Can Co. v. Monsanto, 948 F.2d 1264, 20 USPQ2d 1746 (1991). "Inherency, however, may not be established by probabilities or possibilities. The mere fact that a certain thing *may* result from a given set of circumstances is not sufficient."

APPLICANTS HAVE MET THE LEGAL STANDARD FOR NOVELTY

As discussed supra, the claimed invention is directed to a composition comprising isolated human replicating Kupffer cells, wherein the replicating Kupffer cells express CD68, do not express TGFβ, and at least some of the Kupffer cells have undergone cell division during culture *in vitro,* but wherein the composition does not comprise other Kupffer cells that do not express CD68 and that express TGFβ. In one embodiment, Applicants use limiting dilution methodology to isolate a clonal population of replicating Kupffer cells.

The Prior Art Does Not Teach Every Limitation Of The Claimed Invention

(i) Pulford et al.

Pulford teaches isolation of mouse Kupffer cells, and the growth of these cells in culture. The mouse Kupffer cells in Pulford are a mixed population of cell as exemplified by the appearance of epithelioid cells, multinucleate cells and giant cells in their cultures after a few days (page 69 and Figs 1 and 2).

However, Pulford does not teach isolated human replicating Kupffer cells that express CD68 and do not express TGFβ. Since Pulford does not teach *every aspect* of the claimed invention, it references cannot form the basis for an anticipation rejection under §102(b).

(ii) Gendrault et al.

Gendrault teaches isolation of Kupffer cells from human subjects with liver cancer and co-culture of the Kupffer cells with CEM cells that had been infected with HIV. Gendrault noted syncytia formation and infection of the Kupffer cells with HIV. The Kupffer cells isolated by Gendrault are a mixed population. Gendrault states that the protocol of Kirn et al. (**Exhibit 1**) was used to isolate Kupffer cells. Kirn et al. note that the Kupffer cell population isolated by the protocol they describe is less than 100% pure, and that 10-15% of cells do not show typical features of Kupffer cells (p. 217). Furthermore, Gendrault does not show whether or not Kupffer cells isolated by its methods are replicating. As discussed *infra*, not all Kupffer cells are replicating.

Thus, Gendrault does not teach isolated human replicating Kupffer cells that express CD68 and do not express TGFβ. Since Gendrault does not teach *every aspect* of the

claimed invention, it references cannot form the basis for an anticipation rejection under §102(b).

(iii) Yoshioka et al.

Yoshioka teaches isolation of bovine Kupffer cells. The bovine Kupffer cells in Yoshioka are a mixed population of cells as indicated by the author "the primary cultured cells. . . were alive for one week by changing the medium every day. However, a few dividing cells (probably fibroblast or endothelial cells) were increased after five days."

However, Yoshioka does not teach isolated human replicating Kupffer cells that express CD68 and do not express TGFβ. Since Yoshioka does not teach *every aspect* of the claimed invention, it references cannot form the basis for an anticipation rejection under §102(b).

The Prior Art Reference Does Not Inherently Teach The Claimed Invention

As stated in MPEP §706.02(IV), "...for anticipation under 35 U.S.C. §102, the reference must teach every aspect of the claimed invention either explicitly or impliedly. Any feature not directly taught must be inherently present."

As discussed *supra*, the claimed invention is directed to a composition comprising isolated human replicating Kupffer cells, wherein the replicating Kupffer cells express CD68, do not express TGFβ, and but wherein the composition does not comprise other Kupffer cells that do not express CD68 and that express TGFβ. In one embodiment, the isolated human replicating Kupffer cells have undergone at least one cell division during culture *in vitro*.

However, Kupffer cells are a heterogeneous group of cells (**Exhibit 2**, Abstract; **Exhibit 3**, p. 666, right column, second full paragraph). Further, CD68 is a marker for activated Kupffer cells (**Exhibit 4**, right column, second full paragraph). Therefore, Kupffer cells in general would not necessarily express CD68 and therefore expression of CD68 is not inherent in Kupffer cells in general. Additionally, Kupffer cells do not necessarily replicate in vitro or in vivo (**Exhibit 3**, page 669, left column, last paragraph, and page 670, Table 2; and **Exhibit 5**, page 360, left column, top paragraph). Thus, Kupffer cells are not inherently replicating, *in vivo* or *in vitro*. Accordingly, the prior art references do not inherently anticipate the claimed invention.

Since the prior art references do not teach each and every element of the claimed invention, nor do the prior art references inherently teach the claimed invention, Applicants respectfully request that the Office reconsider and withdraw the rejections to claims under 35 U.S.C. §102(b).

CONCLUSION

If a telephone interview would be of assistance in advancing the prosecution of the subject application, Applicants' undersigned attorney invites the Examiner to telephone her at the number provided below.

No fee, other than $60.00 fee for one-month extension of time, is deemed necessary in connection with the filing of this response. If any further fee is necessary, the Patent Office is authorized to charge any additional fee to Deposit Account No. 50-0306.

Respectfully submitted,

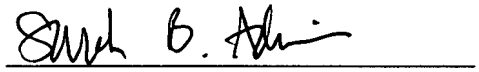

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EXHIBIT 1

U.S. Serial No. 09/991,583

André Kirn et al.,

Hepatology, 1982, 2:216-22

0270-9139/82/0202-0216$02.00/0
HEPATOLOGY

Vol. 2, No. 2, p. 216, 1982
Printed in U.S.A.

Endocytic Capacities of Kupffer Cells Isolated from the Human Adult Liver

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Kupffer cells isolated from human adult livers and maintained in culture display the main structural features of macrophages. They are able to phagocytose latex particles and opsonized sheep erythrocytes *in vitro*. Vaccinia virus as well as Frog Virus 3 are taken up and uncoated in cultured Kupffer cells. Such a model may be used advantageously for studying different problems in liver physiopathology.

Over the last few years, attention has been focused on liver sinusoidal cells, especially Kupffer cells, and their possible role in the pathogenesis of liver disease (1, 2). Thanks to the use of perfusion fixation, our knowledge of the structure of the liver sinusoids under normal and pathological conditions has considerably progressed (3). Several methods have been developed for isolating Kupffer cells from the rat liver (4–6). However, in order to study the part played by the Kupffer cells in human diseases, it is important that the cells be of human origin. We have recently shown that it is possible to isolate viable Kupffer cells from the human adult liver (7). In this paper, we demonstrate that these cells retain their phagocytic capacities *in vitro* and suggest that they may thus provide a good experimental model for studying different problems in liver physiopathology.

MATERIALS AND METHODS

ISOLATION AND CULTURE OF HUMAN KUPFFER CELLS

Five human livers were taken on different occasions from normal adult donors with flat EEGs. Immediately after ablation, the livers were perfused with "Euro Collins" buffer in order to wash out the blood and to minimize contamination with peripheral blood cells. The livers were then rapidly transported to the laboratory in isothermic recipients at +4°C. At the laboratory, a fraction of liver was removed and frozen in liquid nitrogen. The parenchyma of the remaining portion was injected at different points with 0.05% collagenase (Worthington Biochemical Corp., Freehold, N.J.) diluted in Gey's buffer. Pieces of liver were then cut up with scissors and incubated in 0.05% collagenase for 45 min, which leads to the dissociation of the sinusoidal cells. Lysis of the contaminating hepatocytes was obtained by incubation in a New Brunswick gyrotory water bath at a speed of 280 rpm. It has been shown that contaminating parenchymal cells of the rat liver are very fragile and may easily be disrupted (8). The cellular suspension was then filtered on nylon, washed, and suspended in metrizamide (final density, 1.089 gm per cm^3). After 15 min of centrifugation at 1,400 *g*, the cellular band at the interphase was collected, washed, and introduced into a Beckman JE 6 elutriation rotor at 2,500 rpm at a flow rate of 15 ml per min. The increase in the flow rate to 22.4 ml per min enabled a first fraction containing mainly endothelial cells to be collected; with a flow rate of 42.4 ml per min, a second fraction of Kupffer cells was obtained. Fraction II contained between 300 and 500 million cells per elutriation cycle. The viability of the cells, estimated by the absence of trypan blue coloration, varied from 90 to 95% depending on the experiment.

In order to evaluate the contamination with peripheral blood cells, several specimens of liver were defrozen, incubated with collagenase, and the amount of hemoglobin was measured as previously described (6). From the mean value found (96 μg per gm liver), it could be calculated that the monocyte contamination did not exceed 2 per 10^6 Kupffer cells in perfused livers.

The Kupffer cells were cultured in Dulbecco-Hepes medium supplemented with 20% of the donor's serum, either in plastic microplaques (Costar, Data Packaging, Cambridge, Mass.) or in glass tissue culture chambers (Miles Laboratories, Inc., Elkhart, Ind.). Twenty-four-hour cultures were used for the experiments.

AGENTS TO WHICH KUPFFER CELLS WERE EXPOSED

(a) *Opsonized Sheep Erythrocytes (OSE).* Sheep erythrocytes (1×10^9 ml) were opsonized with antisheep

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erythrocyte IgG (Pasteur Institute, Paris, France) having specificities for both heavy and light IgG chains at a final dilution of 1/5,000. OSE were added to the Kupffer cells at a concentration of 40 erythrocytes per cell in Gey's buffer.

(b) *Latex Particles.* Latex particles (Agar Aids S 130-7) of 1.091 μm mean diameter were diluted in Gey's buffer without serum and added to the cells at a final dilution of 1/10.

(c) *Viruses.* Frog Virus 3 (FV 3) and vaccinia virus were cultured in baby hamster kidney cells at 26° and 37°C, respectively; they were purified by sucrose gradient centrifugation.

Determination of Peroxidase Activity

Peroxidase staining was carried out on fresh cells. The cells were spread over glass slides, dried at room temperature, and stained in buffered diamino-benzidine solution (Sigma Chemical Co., St. Louis, Mo.) at pH 7.4 for 30 min. The staining was followed by a short wash with Gey's buffer.

Electron Microscopy

Transmission Electron Microscopy (TEM). Control cells and infected cells were fixed at room temperature with 2.5% glutaraldehyde and postfixed with 1% osmium tetroxide for 1 hr at 4°C. The cells were then dehydrated and embedded in Epon. Thin sections doubly stained with uranyl acetate and lead citrate were examined at 80 KV under a "Philips EM 300" electron microscope.

Scanning Electron Microscopy (SEM). Fixation was carried out as described above. Dehydration was completed with a graded ethanol series. The slides were then immersed in isoamylacetate, and critical-point drying was carried out using a "Polaron E 3000" apparatus. Fragments of dried samples were sputter-coated with gold. The samples were observed and photographed under a "Philips SEM 501" at 15 KV.

RESULTS AND DISCUSSION

The purity of the Kupffer cell fraction was checked by determination of the peroxidase activity and by transmission electron microscope observations. More than 94% of the cells in Fraction II demonstrated a positive peroxidase reaction whereas 85 to 90% showed typical features of Kupffer cells in SEM (Figure 1).

Although the livers were carefully perfused with buffer, contamination with peripheral blood cells and especially with monocytes cannot be completely excluded. The fact that only very low amounts of hemoglobin were found in the cellular suspension after collagenase digestion demonstrates that monocyte contamination is only slight.

Cultured Kupffer cells exhibit the main structural fea-

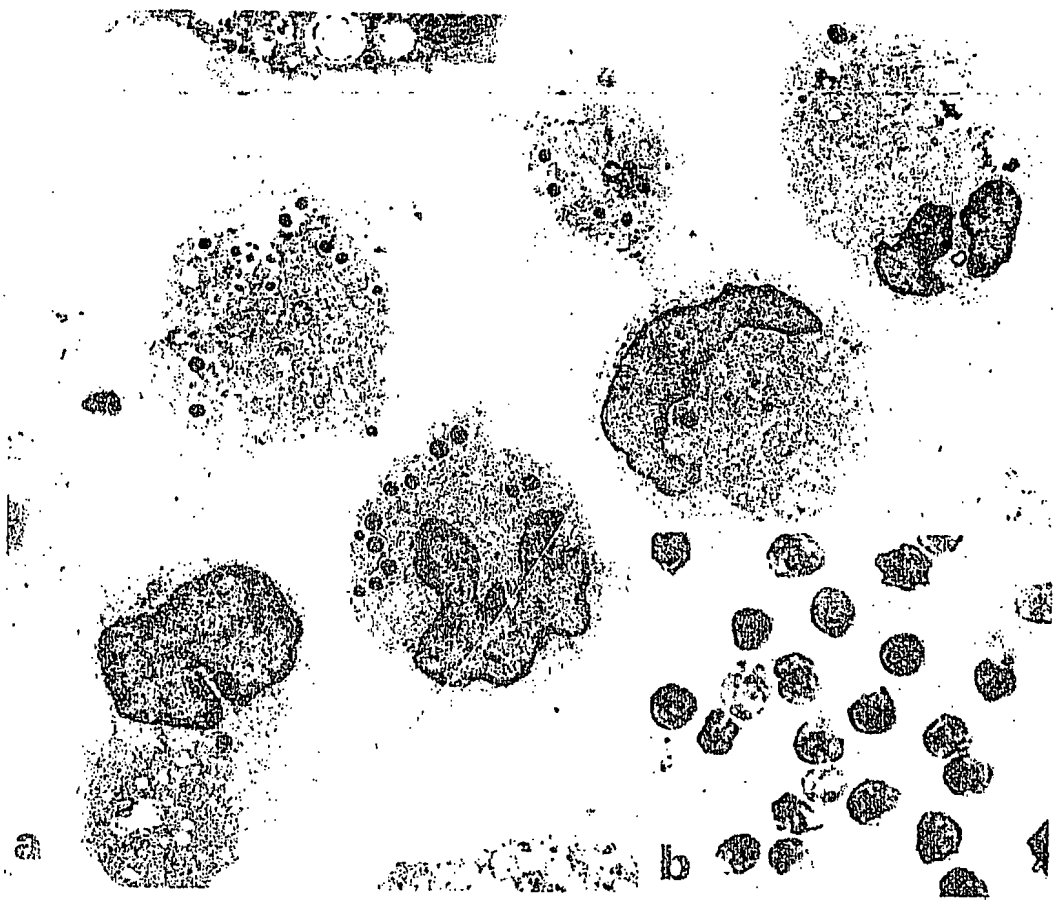

FIG. 1. Human sinusoidal liver cells from elutriation Fraction II. (a) Electron microscopic observation. (b) Peroxidase staining.

tures of macrophages (Figure 2). The surface of the cells is covered with large lamellipodes and microvilli and shows no fuzzy coat. However, it cannot necessarily be concluded that human Kupffer cells in culture are devoid of this fuzzy coat since it has been reported that glutaraldehyde fixation does not allow the preservation of the fuzzy coat of rat Kupffer cells (9). The cytoplasm contains numerous vacuoles and dense bodies which vary in size, density, and content. The mitochondria appear round in tranverse sections and oblong in longitudinal ones. Rough endoplasmic reticulum is distributed throughout the cytoplasm. Human Kupffer cells thus display most of the characteristics reported for rat Kupffer cells (10).

The endocytic capacity of cultured human Kupffer cells was first studied using latex as the "phagocytable" material. A suspension of latex particles was added to the culture which was incubated at 37°C for 60 min. The cells were then washed and processed for TEM. It is clear from Figure 3 that the latex grains have been taken up by the Kupffer cells; several particles may be observed lying inside cytoplasmic vacuoles. In some cases, the phagosome membrane surrounding the particle is clearly visible; in other cases, fusion between lysosomes and latex-containing phagosomes could be observed (results not shown). It should also be pointed out that, because of a marked reduction in the number of microvilli, the cell surface appears much smoother than in nonphagocytosing cells. The smoothing of the phagocytosing Kupffer cell surface has also been observed *in vivo* after the inoculation of glutaraldehyde-treated erythrocytes into the portal vein of the rat (11).

We then studied the uptake of OSE. Figure 4 shows a thin section of a Kupffer cell incubated *in vitro* for 2(min with a suspension of OSE. The OSE attach either t(filipodia or to the surface of the Kupffer cell and, in both cases, bear obvious distortions. One erythrocyte has already been ingested and lies in a vacuole inside th cytoplasm. A particularly striking illustration of the re sult of ingestion is provided by the SEM micrograph i Figure 5; the Kupffer cell spread out on the plasti support is completely misshapen, and its outline bulge with the internalized OSE. At least two conclusions ma be drawn from the fact that human Kupffer cells are abl to phagocytose OSE *in vitro*: (i) the cells still possess F receptors which are responsible for the attachment OSE (12); (ii) they satisfy the metabolic necessities f ingestion to take place (12).

In the last place, we studied the capacity of huma Kupffer cells to interact with viruses *in vitro*. When t cells were infected for 1 hr with vaccinia virus (Figure numerous particles, easily recognizable by their brick ovoid-shaped outlines, could be found lying within t

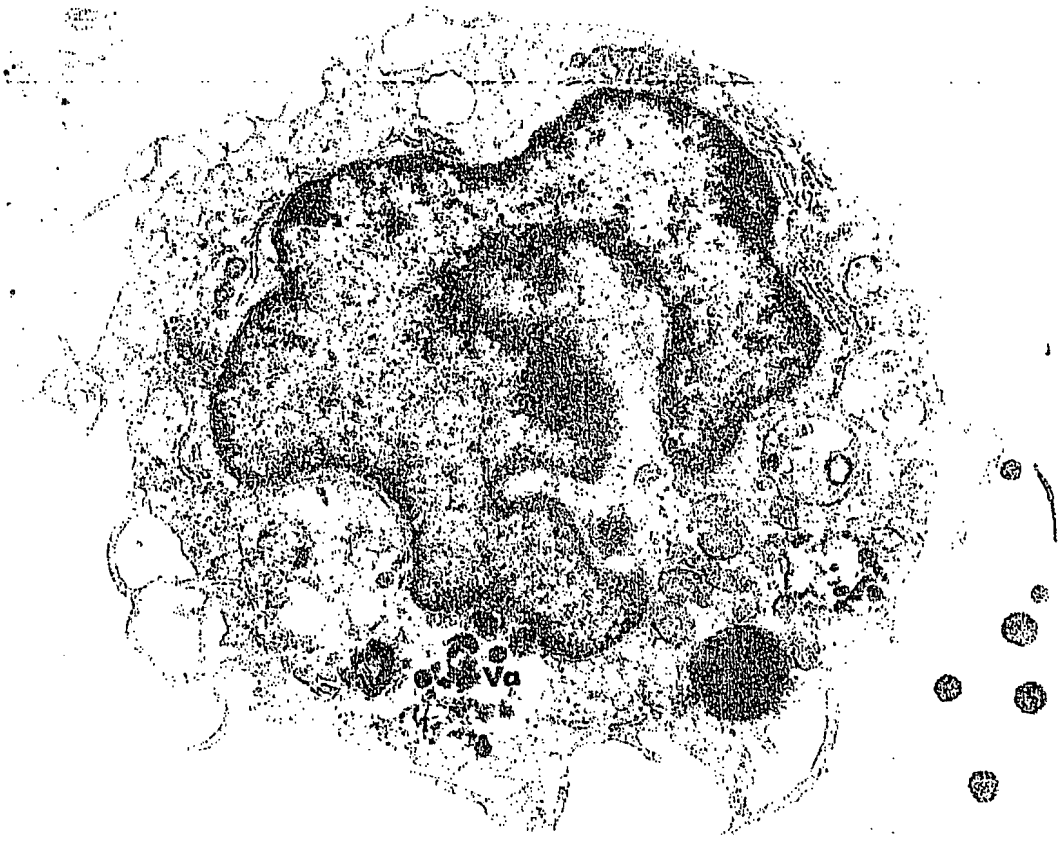

FIG. 2. Kupffer cell isolated from a human adult liver and maintained in culture at 37°C for 24 hr. The cell surface bears numerous mic the cytoplasm displays typical vacuoles (Va). × 12,600.

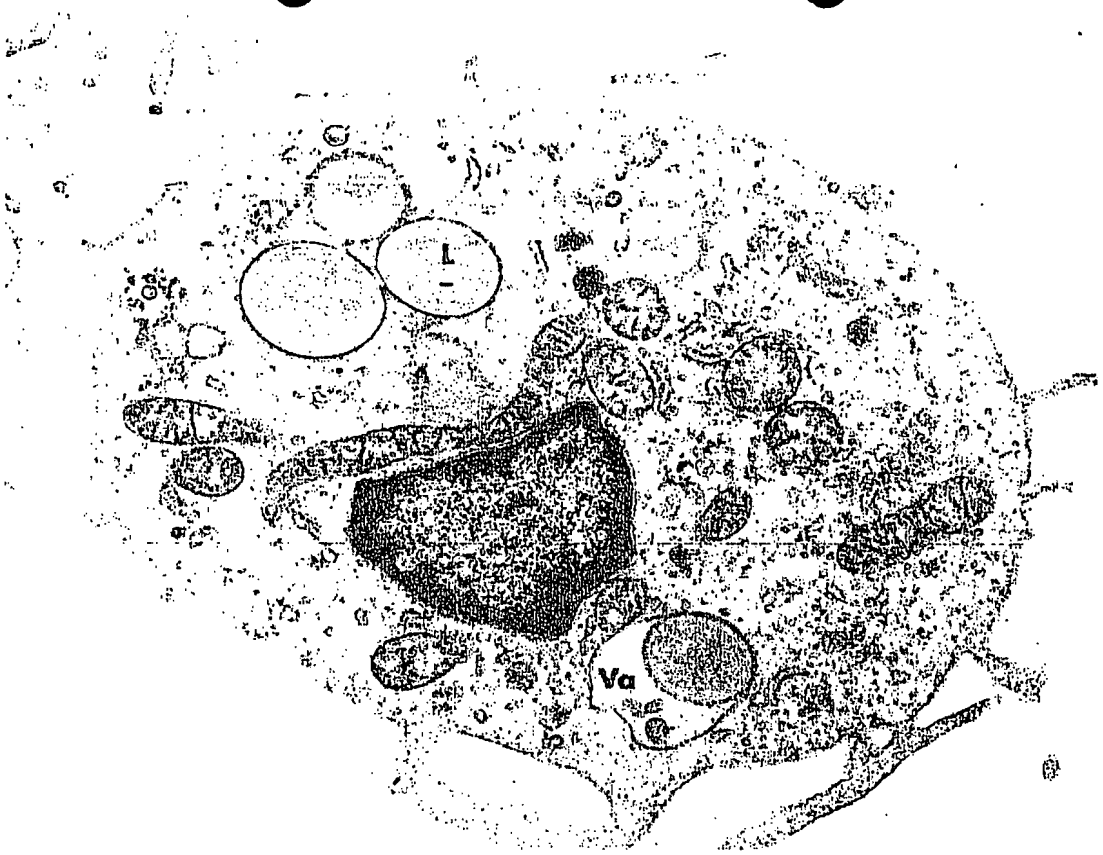

FIG. 3. Phagocytosis of latex particles (L) by human Kupffer cells *in vitro*. Four latex particles (1.091 μm diameter) are enclosed in phagocytic vacuoles (Va). × 15,680.

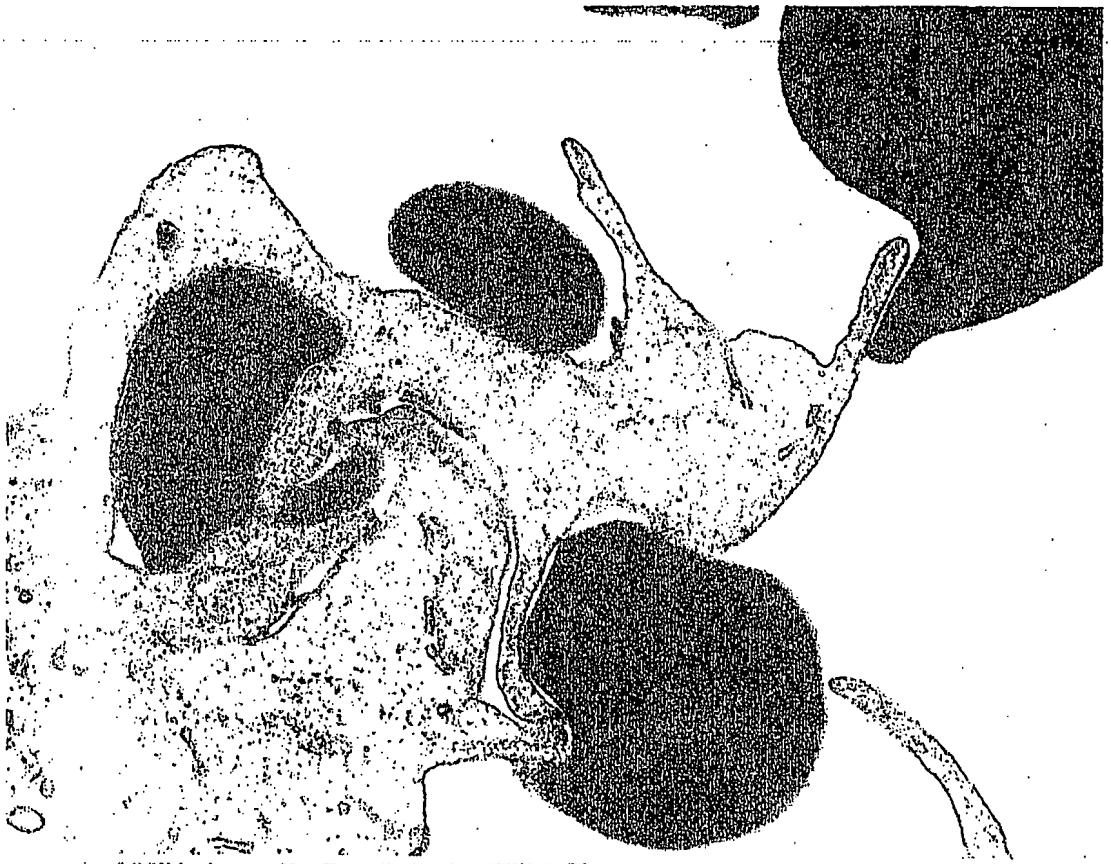

FIG. 4. Phagocytosis of OSE by human Kupffer cells *in vitro* (TEM). Three erythrocytes are attached to the Kupffer cell whereas one red blood cell has already been ingested. × 18,300.

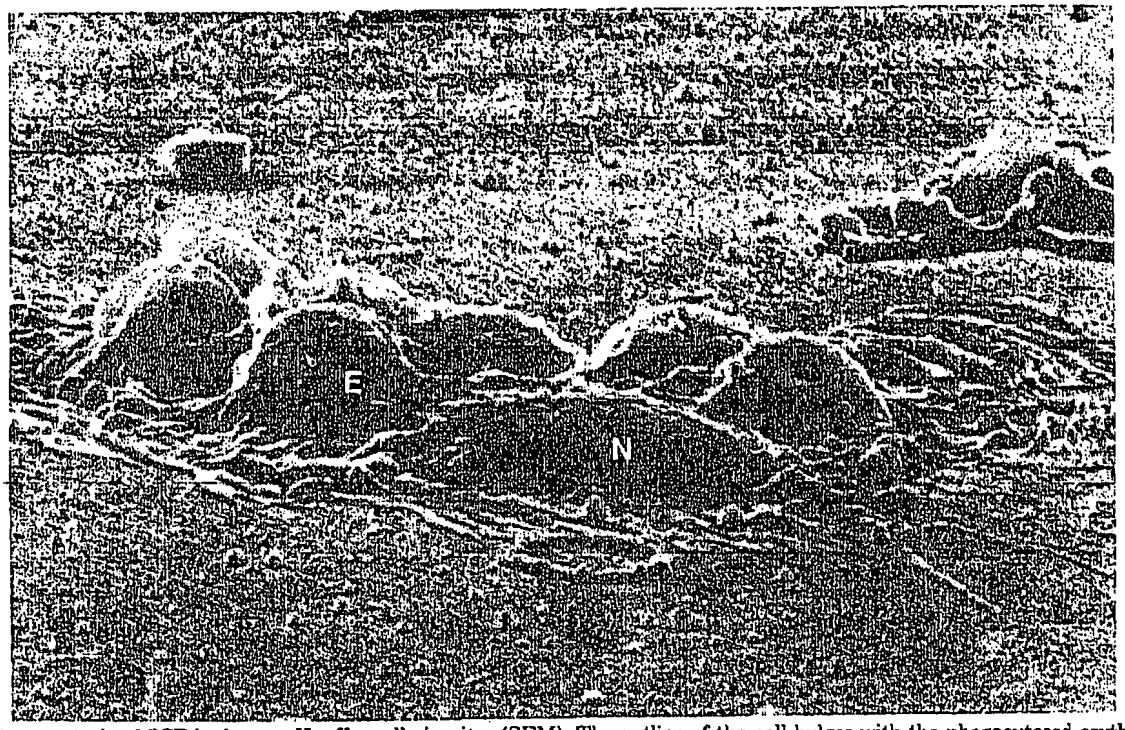

FIG. 5. Phagocytosis of OSE by human Kupffer cells *in vitro* (SEM). The outline of the cell bulges with the phagocytosed erythrocytes (E). The oval protrusion corresponds to the nucleus (N) of the Kupffer cell. × 6,100.

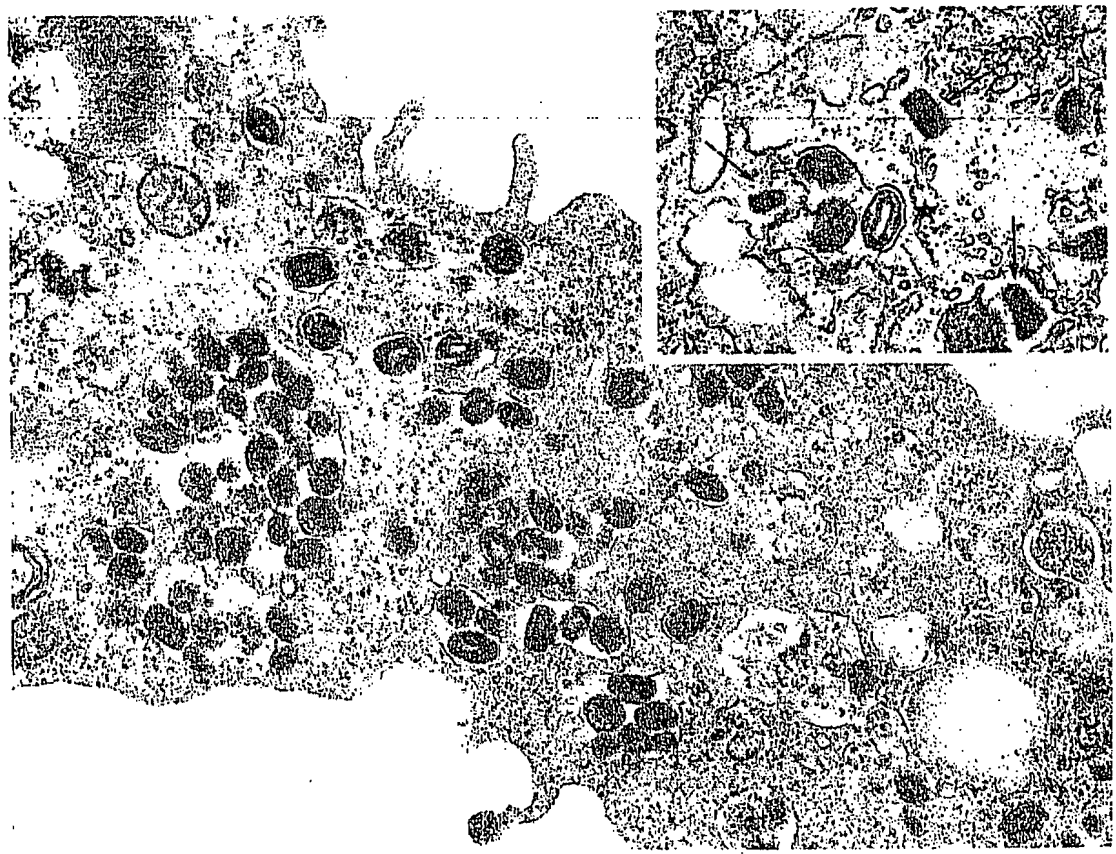

FIG. 6. Human Kupffer cell cultured for 24 hr at 37°C and infected for 1 hr with vaccinia virus. The cytoplasm of the cell displays numerous phagosomes containing viral particles. × 23,200. *Inset*: three viral particles have already undergone the beginning of the uncoating process 15 min after the infection (→); they display rectangular or oval shapes and bear radiating projections, thereby demonstrating that the outer envelope has disappeared (★). Intact virion inside a phagocytic vacuole. × 30,800.

vacuoles inside the cytoplasm. Some vacuoles contain only one or two particles, whereas others are literally filled with virions. Fifteen minutes after the infection, some of the viral particles have already lost their outer envelopes (Figure 6, *inset*), a step which precedes the uncoating process, i.e., the opening of the viral shell allowing the viral DNA to be liberated into the cytoplasm (13). The presence in some cells of "ghosts," which are in fact empty viral capsids (results not shown), indicates that a virus-directed protein synthesis may take place in human Kupffer cells. The synthesis of a specific enzyme indeed is known to be necessary for digestion of the vaccinia virus shell (13).

The interaction of FV 3 with human Kupffer cells is of particular interest since this virus produces an acute degenerative hepatitis in mice and rats starting with sinusoidal cell injury (14). In Kupffer cells infected with FV 3 for 1 hr, numerous hexagonal virus particles may be observed in lucent or dense vacuoles, the latter probably corresponding to phagolysosomes (Figure 7). It should be stressed that the phenomenon of fusion of the viral shell with the membrane of the phagosome, a particular mode of uncoating which occurs in rat Kupffer cells (15, 16), also takes place in human cells (*inset* of Figure 7). This fusion is followed by the release of the viral material into the cytoplasm and by the integration of the viral shell in the membrane of the vacuole, a phenomenon whose possible physiopathological significance is still unknown. Infected Kupffer cells finally degenerate within 3 to 4 hr.

Our results demonstrate that semiperfusion of human livers with collagenase allows viable Kupffer cells to be isolated. The proteases which are often present in commercial collagenase preparations, therefore, seem not to produce any noticeable change in the cells; after incubation in the enzyme solution, the Kupffer cells retain the entirety of their structural and endocytic properties unchanged. Moreover, a similar technique using collagenase recently allowed us not only to isolate endothelial cells from human and rat livers but also to cultivate them for the very first time (17). These cells too retain their morphological features *in vitro* (i.e., well-preserved fenestrae) and adhere to the support which demonstrates that, under our conditions, contaminating proteases do not cause detectable damage to sinusoidal cells.

Among the numerous problems of liver physiopathology which may be tackled using isolated Kupffer cells, three are especially interesting. First, the interaction of human hepatitis viruses with Kupffer cells may be studied. At the present time, it is not known: (i) how hepatitis viruses present in the blood stream reach the parenchymal cells; (ii) if they are able to multiply in the Kupffer cells and if so, whether this step is necessary for infection to occur. Second, the capacity of the Kupffer cells to detoxify endotoxin *in vitro* may be investigated. It has been claimed that the macrophages are responsible for

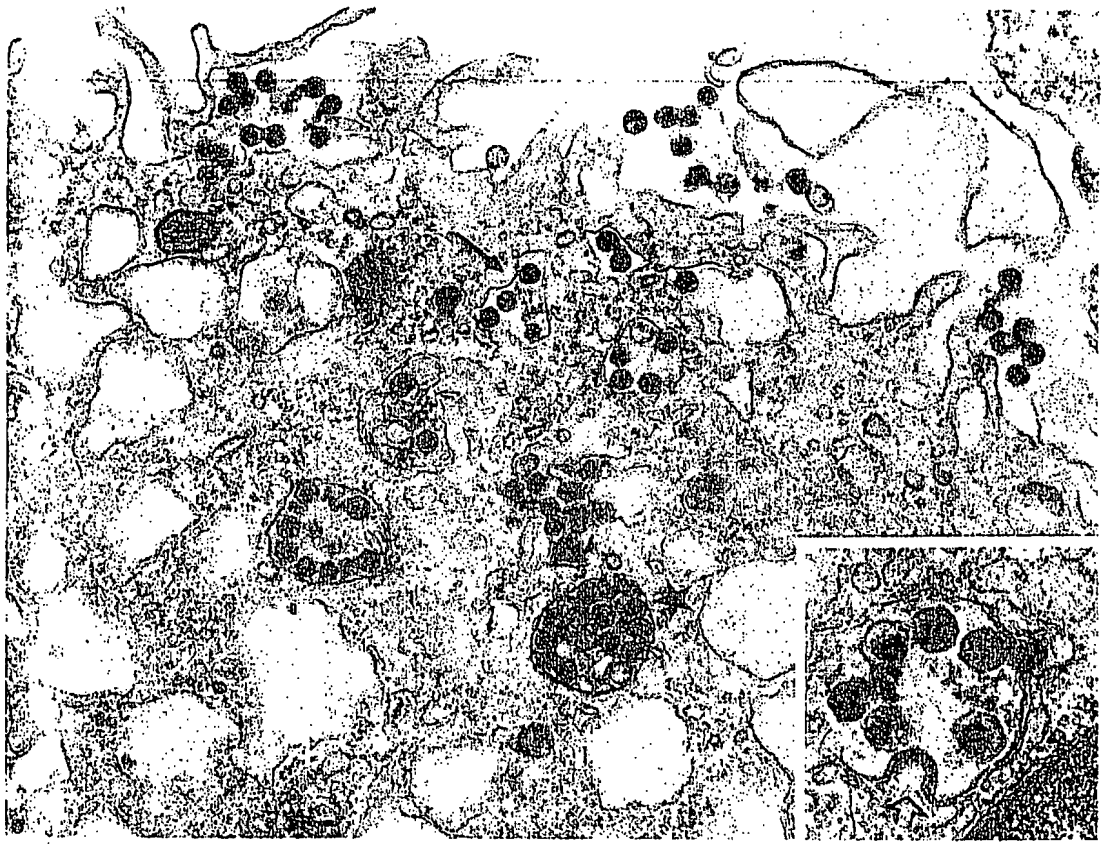

FIG. 7. Human Kupffer cell cultured for 24 hr at 37°C and infected for 30 min with FV 3. Hexagonal viral particles may be observed in lucent vacuoles situated near the cell membrane (→); some other vacuoles containing viruses (★) display a dense content. × 23,200. *Inset*: fusion of the virus shell with the membrane of a vacuole; the content of the virion has been liberated into the cytoplasm of the Kupffer cell (⇨). × 41,500.

the detoxification of the endotoxins (18) but this has never been demonstrated experimentally for Kupffer cells. Third, isolated Kupffer cells may constitute an excellent model for screening the ability of drugs to produce an activation or a depression of the reticuloendothelial system. It is important that drugs capable of modifying Kupffer cell activity be recognized.

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EXHIBIT 2

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Review

Tri-iodothyronine differentially induces Kupffer cell ED1/ED2 subpopulations

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Abstract

Thyroid calorigenesis is carried out by activation of cytochrome-c oxidase, as well as by induction of mitochondrial and nuclear genes that code for cell respiratory apparatus components and uncoupling proteins. These effects operate increments in basal metabolic rate and also lead to increased production of oxygen and nitrogen reactive species in liver parenchymal cells. The hepatic antioxidant system is also compromised, since superoxide dismutase and catalase activities, glutathione content and lipid soluble antioxidants are reduced. Liver macrophages contribute to the hepatic oxidative stress observed in T_3-treated rats, and both Kupffer cell hyperplasia and hypertrophy are reported. Kupffer cells constitute the main fixed macrophage population in the body and are a heterogeneous group of cells, derived from a less numerous population of local precursors, which are morphologically fairly distinguishable from the mature lineage elements. ED1 and ED2 antigens have been particularly useful in the characterization of Kupffer cell subpopulations. In particular, antibodies against these antigens provided evidence that T_3-induced Kupffer cell hyperplasia causes a shift on liver macrophage population phenotype, leaning towards younger cell types. Despite the fact that sinusoidal environment itself stimulates the proliferation of macrophage precursors and their differentiation into Kupffer cells, increased Kupffer cell turnover rates modify the sinusoidal environment and may imply further functional effects. Thus, Kupffer cell hyperplasia secondary to increased T_3 levels is potentially a pro-inflammatory event, which involves both, the expansion of Kupffer cell precursor population by means of circulating monocyte recruitment,

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and the differentiation of preexisting local Kupffer cell precursors into mature liver macrophages.

Abbreviations: AP-1, activator protein-1; $GdCl_3$, gadolinium chloride; GM-CSF, granulocyte and macrophage-colony stimulating factor; GSH, reduced glutathione; H_2O_2, hydrogen peroxide; IL-3 and IL-6, interleukin-3 and -6; M-CSF macrophage-colony stimulating factor; NF-κB, nuclear factor κB; PAF, platelet activating factor; T_3, 3,5,3′-L-triiodothyronine; TNF-α, tumor necrosis factor α

Keywords: Kupffer cell hyperplasia; ED1; ED2 antigens; Liver macrophages; T_3; Hyperthyroidism; Oxidative stress

Contents

1. Liver oxidative stress and inflammation in hyperthyroidism

The general relationship between hyperthyroidism and oxidative stress was proposed after the establishment of a significant and direct correlation between basal metabolic rate and tissue lipid peroxidation potential in different mammalian species (Cutler, 1985). Hepatic oxidative stress occurs early in experimental animals made hyperthyroid by 3,5,3′-L-triiodothyronine (T_3) administration, even if only subclinical disease is produced. Increments in the production of oxygen (microsomal, mitochondrial, and peroxisomal) and nitrogen (cytosolic) reactive species correlate to the acceleration of aerobic metabolism, evoked by T_3 during its calorigenic action on the liver tissue (Fernández et al., 1985; Videla, 2000). Thyroid calorigenesis is carried out by the activation of cytochrome-c oxidase (short-term pathway), as well as induction of mitochondrial and nuclear genes that code for cell respiratory apparatus components and uncoupling proteins (long-term pathway) (Videla, 2000). Augmented biliary excretion of oxidized glutathione and higher lipid and protein oxidation in the liver were also observed (Tapia et al., 1999; Videla, 2000). The main hepatic antioxidant systems are found compromised, since superoxide dismutase and

catalase activities, and GSH and lipid soluble antioxidants contents are reduced (Fernández et al., 1991; Tapia et al., 1999).

Heart myocytes and liver parenchyma are highly susceptible to T_3 cytotoxicity. Liver tissue initially adapts to the increased protein synthesis by a reversible hepatocyte modification, known as cloudy swelling, which is accompanied by some degree of Kupffer cell hypertrophy (Del Monte, 2001). By the time effective treatment for hyperthyroidism is started, concurrent liver damage is usually found, evidenced by fatty change, centrilobular hepatic necrosis, and cirrhosis.

With the exception of differentiating preadipocytes, extra-thyroidal rat and human tissues are largely irresponsive to thyrotropin (Haraguchi et al., 1996). Subsequently, major metabolic modifications observed in hyperthyroid states are due to the action of thyroid hormone molecules upon tissue targets. However, adipogenesis and adipocyte differentiation potentially contribute to the regulation of inflammatory conditions as seen in thyroid eye disease (Ludgate and Baker, 2002).

2. Effects of T_3 on Kupffer cells

Kupffer cells activity contributes to the hepatic oxidative stress observed in T_3-treated rats. In addition to T_3-induced increase in protein synthesis, an expansion of the Kupffer cell population is reported (Tapia et al., 1997). Kupffer cell hyperplasia is accompanied by an increase in the luminescence emitted by liver tissue homogenates, stimulated by opsonized zymosan (Videla et al., 1995). Perfusion experiments, carried out with livers isolated from rats made hyperthyroid by T_3 administration, showed increases in TNF-α production, oxygen consumption, and Kupffer cell phagocytic capacity (Tapia et al., 1997). In vivo colloidal carbon uptake is also enhanced in the

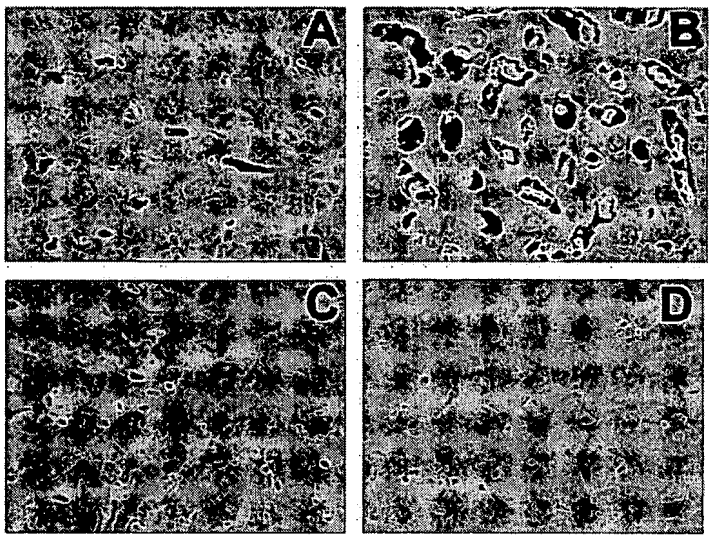

Fig. 1. Representative photomicrography of colloidal carbon uptake by the liver of rats treated with: (A) saline, (B) T_3, (C) $GdCl_3$, (D) T_3 and $GdCl_3$. Formalin fixed, paraffin embedded liver slices (3 μm) were counterstained with nuclear neutral red. Magnification: 400×.

liver of T_3-treated animals (Fig. 1). All the above effects are abolished by gadolinium chloride ($GdCl_3$) administration (Tapia et al., 1997). $GdCl_3$, a selective Kupffer cell inactivator, also significantly reduces GSH consumption, lipid peroxidation, and nitric oxide production by the liver (Hardonk et al., 1992; Tapia et al., 1997).

The pathophysiology of Kupffer cell hyperplasia is not entirely elucidated. A direct action of thyroid hormone, such as that verified in other cell types, as in hepatocytes, has been proposed for local Kupffer cell precursors which proliferate in response to increasing concentrations of T_3 (Columbano and Shinosuka, 1996; Torres et al., 1999). This hypothesis implies a non-inflammatory expansion of the macrophage population, which can induce oxidative stress as a secondary phenomenon. On the other hand, a pro-inflammatory response in the sinusoidal microenvironment could trigger monocyte recruitment from peripheral blood and the production of inflammatory mediators in response to the redox imbalance produced in the liver.

3. Proliferation and differentiation of Kupffer cells

According to the concept of mononuclear phagocytic system, inflammatory exudate macrophages and resident macrophages are all derived from peripheral blood monocytes and differentiated into low proliferative capacity cells (Takezawa et al., 1995). While in the newborn the liver is a central organ for the production and supplementation of macrophages and their precursors to other tissues, in healthy adults only 2% of liver cells are under division (Naito et al., 1997). The elevated proliferative potential of fetal liver macrophages is essential to assure their survival in the liver and the colonization of other fetal tissues, by means of the blood stream (Naito et al., 1997). Nevertheless, Kupffer cell proliferation by local cell division can be observed in adult organisms after injection of macrophage stimulating agents and following partial hepatectomy.

Putative local liver macrophage precursors are proposed to derive from circulating monocytes, which originate in bone marrow hematopoietic tissue (Crofton et al., 1978). Among other growth factors, such as interleukins (IL-6, IL-3) and granulocyte and macrophage-colony stimulating factor (GM-CSF), macrophage-colony stimulating factor (M-CSF) seems the most important stimulator in the development and differentiation of the restricted macrophage lineage (Naito et al., 1997).

4. Kupffer cell subpopulations

Macrophages constitute a functional and morphologically heterogeneous group of cells, and the subgroups may be identified by immunohistochemical techniques. ED1 and ED2 antigens have been particularly useful in the characterization of Kupffer cells subpopulations. ED1 antibody binds to most macrophage populations, as well as peripheral blood monocytes and bone marrow precursors. Despite the fact that a few other cell types also express ED1 when activated, it has been used as a

mononuclear phagocytic system member label (Damoiseaux et al., 1994). ED1 antibody recognizes a simple chain of glycoprotein of 90,000–110,000 kDa, predominantly expressed on the lysosomal membrane and, to a lower extent, on the cell surface. ED1 expression and glycosylation levels are accentuated by phagocytic stimulus. Differential expression of this antigen derives from the exchange of

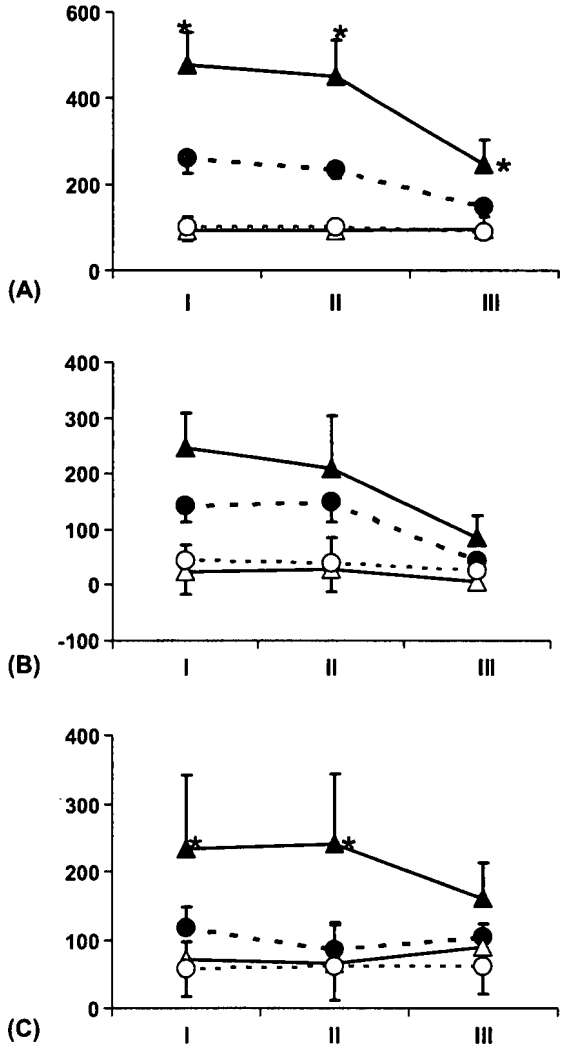

Fig. 2. Macrophage subpopulation cell densities (cells/mm^2) in control and hyperthyroid rat liver regions (I–III). Immunohistochemistry employed ED1 and ED2 MoAbs (Serotec), and was developed with Alkaline Phosphatase Envision System (DAKO). Formalin fixed, paraffin embedded liver slices, 3 μm, were used. Morphometric analysis was sampled out in a 0.7 mm^2 area/ liver region, with Image-Pro® Plus v4 (Micronal), after hematoxylin counterstaining. (A) ED1, (B) ED2, and (C) estimated ED1+/ED2- population (calculated from ED1 and ED2 data). Triangles: T3 treated animals, circles: control animals; open symbols were employed for $GdCl_3$ treated animals in each group. Values in the figures are means ±SD; 6 rats/group. *$p < 0.05$. Statistical differences between control and hyperthyroid groups were analyzed by ANOVA followed by variance stabilization by the Box-Cox procedure.

membrane components between these compartments, since plasma membrane recycling is an important aspect of phagocytosis (Damoiseaux et al., 1994). ED2 antibody recognizes a membrane antigen of resident macrophages in rats. This antibody is largely used to identify Kupffer cells (Dijkstra et al., 1985; Yamate et al., 2001; Ide et al., 2002) and it is specific for resident macrophages.

Two main Kupffer cell subpopulations are distinguishable by the combined use of ED1 and ED2 antibodies. These subpopulations present slightly different morphology, but distinct anatomical distribution and functional characteristics (Armbrust and Ramadori, 1996; Sato et al., 1998; Yamate et al., 1999). One population of small ED1-positive cells is present around the portal triad and centrilobular veins. This population is described in the literature as "small Kupffer cells", "local Kupffer cell precursors", or just taken as the less mature members of the liver macrophage population collectively called "Kupffer cells". Small Kupffer cells are usually not labeled by ED2 antibody. Another population, constituted by large ED1 and ED2-positive macrophages, is placed along the sinusoids. These cells are described as mature hepatic tissue macrophages, often associated to the name "large Kupffer cells" or simply "Kupffer cells" (Armbrust and Ramadori, 1996; Sato et al., 1998; Yamate et al., 1999). Both ED1 and ED2 labeling increase in the liver of hyperthyroid animals (Fig. 2). However, this increment is more pronounced in the ED1+/ED2- subpopulation than in ED1+/ED2+ cells, indicating that T_3-induced Kupffer cell hyperplasia causes a shift on liver macrophage population phenotype, leaning towards younger cell types.

The sinusoidal environment itself stimulates local proliferation of macrophage precursor mononuclear cells and their differentiation into Kupffer cells (Armbrust and Ramadori, 1996). However, increased Kupffer cell turnover rates modify sinusoidal environment and such shift on liver macrophage population in T_3-treated animals may add to a pro-inflammatory condition. For instance, recently migrated monocytes are probably activated during their passage through the vascular wall, producing reactive species (Decker, 1997).

Furthermore, Kupffer cell hyperplasia implies functional effects that may depend on cell activation. The silent clearance of inflammation promoters, carried out mainly by macrophages, is an anti-inflammatory action (Witmer-Pack et al., 1993). Regardless great phagocytic capacity, steady state Kupffer cells present small production of oxygen and nitrogen reactive species and low expression of class II histocompatibility receptors (MHC) (Bowens et al., 1992; Armbrust and Ramadori, 1996). They can, however, trigger inflammatory response, including antigen presentation (Naito et al., 1997).

5. Concluding remarks

Kupffer cell activation results in mononuclear cell recruitment from circulation, receptor expression, and the secretion of several molecules, such as enzymes, eicosanoids, PAF, cytokines, complement proteins, oxygen and nitrogen reactive species, and apolipoprotein *E* (Winwood and Arthur, 1998; Armbrust and Ramadori, 1996). Sinusoidal endothelium and Ito cells respond to factors released by

activated Kupffer cells, thus amplifying the spectrum of released substances during inflammation. This joint action triggers heat shock protein synthesis and liver regeneration responses, since some of the produced oxidizing agents, i.e. H_2O_2, can activate transcription factors (NF-κB, AP-1) and shift the balance between death signs and liver cell proliferation (Sen and Packer, 1996). In conclusion, Kupffer cell hyperplasia secondary to increased T_3 levels is a potentially pro-inflammatory event, which involves both the of Kupffer cell precursor population expansion, through circulating monocyte recruitment, and the differentiation of local Kupffer cell precursors into mature liver macrophages.

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EXHIBIT 3

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Proliferation of Rat Liver Macrophages *In Vitro*: Influence of Hemopoietic Growth Factors

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We examined the effects of several hemopoietic growth factors on proliferation of rat liver macrophages *in vitro*. The proliferative response of liver macrophages to hemopoietic growth factors was assayed on the basis of [methyl-^{3}H]thymidine uptake. Macrophage colony-stimulating factor and recombinant murine granulocyte-macrophage colony-stimulating factor stimulated [methyl-^{3}H]thymidine incorporation in a concentration-dependent manner. With granulocyte-macrophage colony-stimulating factor, maximum incorporation was observed at 50 U/ml, whereas with macrophage colony-stimulating factor no incorporation plateau was observed up to 50% L929-conditioned medium. Incubation of liver macrophages with various concentrations of recombinant human interleukin-2, recombinant murine interleukin-3 and recombinant human interleukin-6 or culture medium alone did not result in significant incorporation of [methyl-^{3}H]thymidine. When liver macrophages were fractionated according to cell size, highest incorporation was observed in the large macrophages. Proliferating cells in cultures of all subfractions were microscopically identified as typical macrophages by the use of macrophage-specific monoclonal antibodies. After 6 days in culture, these macrophages had functional properties similar to those of resident liver macrophages with respect to phagocytosis and *in vitro* activation with immunomodulators to tumorcytotoxicity and secretion of nitric oxide and tumor necrosis factor-α. These results suggest that macrophage colony-stimulating factor and granulocyte-macrophage colony-stimulating factor play important roles among the regulatory factors that support local proliferation of rat liver macrophages. (HEPATOLOGY 1994; 19:666-674.)

The mononuclear phagocyte or macrophage has long been recognized as a key cell in the mammalian host defense system. It plays an important role in host immune responses to infection and neoplastic diseases and is a major source of inflammatory and growth-related cytokines (1-5). The macrophages of the liver (Kupffer cells) represent the largest population of fixed mononuclear phagocytes in the body (6). The origin and proliferative capacity of liver macrophages under normal conditions is still subject to considerable debate. According to the concept of the mononuclear phagocyte system, resident macrophages are thought to be derived from blood monocytes and are short-lived, terminally differentiated cells with little capacity to proliferate (7-9). Others, however, maintain the view that resident liver macrophages can proliferate locally and survive by self-renewal (6).

Although local proliferation of resident liver macrophages has been shown to be an important component in maintenance and expansion of the liver macrophage population (10-13), little is known about the factors involved in multiplication of these cells. Several cytokines—including macrophage colony-stimulating factor (M-CSF or CSF-1), granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin-2 (IL-2), interleukin-3 (IL-3) and interleukin-6 (IL-6) (14-21)—have been shown to induce proliferation of macrophages or their precursors in bone marrow and various other organs, both *in vitro* and *in vivo*. It is now clear that a hierarchy of hemopoietic growth factors acting during different stages of macrophage differentiation exists (14, 22).

The liver macrophage population is a heterogeneous population of cells. Differences in functional characteristics of subpopulations of liver macrophages, differing in cell size, have been observed with respect to phagocytic capacity, lysosomal enzyme activity, *in vitro* tumorcytotoxicity, tumor necrosis factor-α (TNF-α) secretion, IL-1 secretion and their localization in the liver acinus (23-26). Differences in cell size presumably reflect the degree of differentiation of the cells.

In this report, we describe the effects of several hemopoietic growth factors on proliferation of rat liver macrophages *in vitro*. The proliferative capacity was determined for the whole liver macrophage population and for subclasses of liver macrophages, differing in cell size. Additionally, different effector functions of the

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proliferated macrophages were determined; phagocytic capacity was determined on the basis of uptake of latex particles, and cytotoxic properties were determined on the basis of *in vitro* activation to tumor cytotoxicity and induction of secretion of nitric oxide (NO) and TNF-α.

MATERIALS AND METHODS

Animals. Male Wag/Rij rats (TNO, Rijswijk, The Netherlands), 10 to 12 wk old and weighing 180 to 220 gm, were used. They were given laboratory chow and water *ad libitum*. Animal protocols were approved by the Ethical Committee of the Rijksuniversiteit of Groningen.

Culture Medium. Cells were cultured in RPMI-1640 (Gibco Laboratories, Grand Island, NY) supplemented with 2 mmol/L L-glutamine (Flow Laboratories, Irvine, CA), penicillin G (100 U/ml), streptomycin (100 μg/ml) (both from Gist-Brocades, Delft, The Netherlands) and 10% heat-inactivated FCS (Gibco Laboratories).

Reagents. Muramyl dipeptide (MDP) was a generous gift of Ciba-Geigy (Basel, Switzerland). Lipopolysaccharide B from *Escherichia coli* 0127:B8 (LPS) was purchased from Difco Laboratories (Detroit, MI). [Methyl-^{3}H]thymidine ([^{3}H]dThd; specific activity 5 Ci/mmol) was from Amersham International (Little Chalfont, Buckinghamshire, UK). Monoclonal antibodies ED1 and ED2, recognizing rat macrophages (27), were generous gifts of Dr. C.D. Dijkstra (Free University of Amsterdam). The anti-BrdUrd monoclonal antibody MoBu-1 (28) was a generous gift of Dr. L. de Ley (University of Groningen). 5 Bromodeoxyuridine (BrdUrd) was from Serva (Heidelberg, Germany).

Growth Factors. Recombinant mouse GM-CSF (specific activity 1×10^7 U/mg), recombinant human IL-6 (specific activity 2×10^6 U/mg) and recombinant mouse TNF-α (specific activity 6×10^6 U/mg) were all purchased from Boehringer (Mannheim, Germany). Recombinant human IL-2 (specific activity 18×10^6 U/mg) was from Eurocetus BV (Leiden, The Netherlands), and recombinant mouse IL-3 (specific activity 1×10^6 U/mg) from Genzyme (Boston, MA). L929-conditioned medium (L929-CM) was used as a source of CSF-1 (M-CSF) (18). The mouse L929 cell line was cultured as a confluent monolayer for 4 days in culture medium. The supernatant was collected and centrifuged for 10 min at 600 *g* to remove cells, sterile-filtered through a 0.22-μm filter (Millipore Corp., Bedford, MA) and frozen in suitable aliquots at −20° C until use.

Tumor Cell Culture. C26 colon adenocarcinoma cells, syngeneic with BALB/c mice, were grown in culture medium at 37° C in a humidified atmosphere with 5% CO_2 in air.

Isolation of Rat Liver Macrophages. Rat liver macrophages were isolated by means of pronase digestion of the liver as described before (29). The liver was perfused first with Gey's balanced salt solution (GBSS) to remove blood and then for 3 to 5 min with 0.05% pronase. The liver was removed, cut in small pieces and incubated for 45 min at 37° C in the presence of DNase (0.8 μg/ml). Subsequent isolation procedures were performed at 4° C. The cell suspension was washed once, and nonparenchymal cells were separated from nonviable parenchymal cells and remaining erythrocytes by means of centrifugation on a Nycodenz (Nycomed, Oslo, Norway) gradient for 15 min at 1,500 *g*. The top layer, containing the nonparenchymal cells, was washed once and resuspended in 5 ml of GBSS containing DNase. The nonparenchymal cell suspension was flushed into an elutriation rotor (type JE-6 elutriation rotor; Beckman Instruments, Palo Alto, CA) at 4° C with a flow rate of 23.0 ml/min and a rotor speed of 2,500 rpm. At this flow rate, lymphocytes, including natural killer cells, and endothelial cells were flushed out in 250 ml of GBSS. The macrophages were collected at a flow rate of 46.5 ml/min in 150 ml of GBSS, concentrated by means of centrifugation at 700 *g* for 10 min and resuspended in culture medium. In some experiments the total macrophage population was separated into four subfractions. The four subfractions (A through D) of liver macrophages were collected in 100-ml quantities each at flow rates of (A) 25, (B) 30, (C) 35 and (D) 46.5 ml/min. At least 90% of the isolated cells were judged to be macrophages on the basis of structure and nonspecific esterase staining.

Proliferation Assay. Incorporation of [^{3}H]dThd was used to assess cell proliferation. Liver macrophages (4×10^4 cells/well), distributed onto 96-well flat-bottomed tissue culture plates, were incubated in 200 μl of culture medium with or without growth factors at 37° C in 5% CO_2 and air. After various periods of incubation, 0.5 μCi of [^{3}H]dThd was added to the wells, and the cells were incubated for another 20 hr. Cells were harvested on glass-fiber filters with a semiautomatic cell harvester, and [^{3}H]dThd incorporated in cellular DNA was counted in 2 ml of scintillation solution.

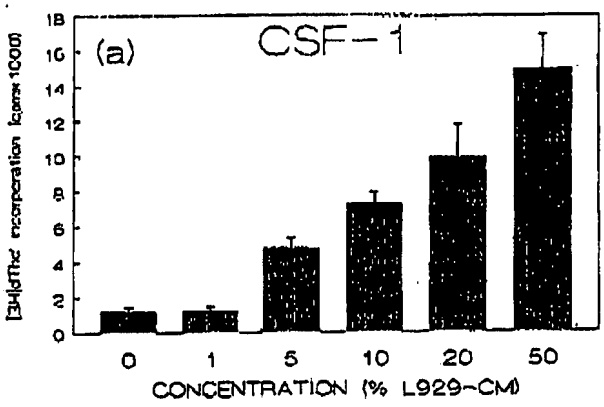
Characterization and detection of proliferating cells. Liver macrophages (4×10^4), plated onto 96-well plates, were incubated in 200 μl of culture medium with CSF-1 (20% L929-CM), GM-CSF (50 U/ml) or medium for different time periods. BrdUrd (10 μmol/L) was added to each well, and cells were incubated for an additional 20 to 24 hr. Cells were removed by means of incubation with 0.03 mol/L EDTA and washed with PBS. The resulting cell suspension was centrifuged in a cytocentrifuge. The slide glass was dried and fixed with acetone for 10 min, dried again and stored at −20° C. Within 3 wk, the slides were subjected to immunohistochemical staining with monoclonal antibodies ED1 and ED2, in combination with doublestaining with MoBu-1, as described by Harms et al. (28).

Macrophage-mediated Cytolysis Assay. Macrophage-mediated cytolysis was assessed with a radioactivity release assay described previously (29). Isolated liver macrophages were grown in culture flasks in culture medium containing CSF-1 (20% L929-CM) or GM-CSF (50 U/ml). After 6 days, the medium was discarded and the cells were removed by means of addition of 0.03 mol/L EDTA and washed twice in PBS. Then, 2×10^5 macrophages/well were seeded onto 96-well plates and incubated in culture medium with or without immunomodulators, together with 10^4 [^{3}H]dThd-labeled C26 adenocarcinoma tumor cells (macrophage/tumor cell ratio, 20:1). Radiolabeled tumor cells were also plated alone as a control for spontaneous release of label from the tumor cells. After 48 hr of coculture, the supernatants were collected, and radioactivity was measured in a liquid scintillation counter. We determined total radioactivity added per well by measuring radioactivity of 10^4 tumor cells in 50 μl of medium mixed with 25 μl of 10% SDS. Cytolysis was calculated as follows:

$$\% \text{ Cytolysis} = 100 \times \frac{a - b}{c - b}\%$$

in which *a* is disintegrations per minute in the supernatant of tumor cells cocultured with test macrophages, *b* is disintegrations per minute in the supernatant of tumor cells cultured alone and *c* is disintegrations per minute in the total amount of tumor cells added per well.

Determination of NO secretion. Nitrite content, secreted into the medium during a 24-hr period after activation, was determined as a measure of total NO production by means of the microplate assay method of Ding et al. (30). Briefly, 100 μl samples were incubated with an equal volume of Griess



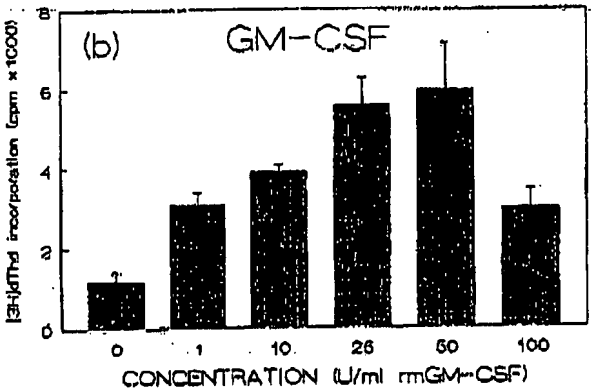


FIG. 1. Effect of concentration of (a) CSF-1 and (b) GM-CSF on the incorporation of [^{3}H]dThd by rat liver macrophages *in vitro*. Data are expressed as mean counts per minute ± S.E.M. of one representative experiment; they were obtained as described in the legend of Table 1.

TABLE 1. Effect of hemopoietic growth factors on proliferation of rat liver macrophages *in vitro*

Culture condition	Incorporation of ^{3}H-dThd (counts per minute)
Medium	2,080 ± 612[a]
CSF-1 (20% L929-CM)	18,380 ± 5,240[b]
Recombinant murine GM-CSF (50 U/ml)	8,476 ± 1,191[b]
Recombinant human IL-2 (U/ml)	
1	2,127 ± 683
10	3,262 ± 1,238
100	3,274 ± 1,138
500	3,262 ± 1,238
Recombinant murine IL-3 (U/ml)	
1	2,105 ± 707
10	1,998 ± 686
100	2,068 ± 703
250	1,982 ± 755
Recombinant human IL-6 (U/ml)	
1	1,765 ± 493
10	1,752 ± 428
100	2,004 ± 583
1,000	1,618 ± 472

A total of 4×10^4 liver macrophages/well were incubated with or without various concentrations of hemopoietic growth factors for 6 days. Twenty hours before the termination of culture, 0.5 μCi of ^{3}Hd'Thd was added to each well.

[a] Data expressed as the mean counts per minute ± S.E.M. of three independent experiments performed in triplicate.

[b] $p < 0.05$ vs medium alone.

reagent (1% sulfanilamide, 0.1% naphthylethylenediamine dihydrochloride, 2.5% H_3PO_4) at room temperature for 10 min. Absorbance at 550 nm was measured with a microplate reader. The nitrite concentration was determined with a sodium nitrite standard curve. All reagents were purchased from Sigma Chemical Co.

Determination of TNF-α Secretion. TNF-α, secreted into the medium during a 24-hr period after activation, was determined with the L929-lysis assay (31). Briefly, 4×10^4 L929 cells were grown in 96-well flat-bottomed microtiter plates in the presence of 1 μg/ml actinomycin D (Sigma Chemical Co.) and serial twofold dilutions of test samples in a total of 200 μl volume. After 18 hr of incubation at 37° C, 25 μl of glutaraldehyde (25% vol/vol; Sigma Chemical Co.) was added to the wells for fixation of cells attached to the plastic. After 15 min, the supernatant was discarded and the plates were rinsed with tap water. Subsequently, cells were stained with 0.05% methylene blue (in aqua dest) for 20 min. After repeated washing of the plates, the adherent cells were dissolved in 0.33 N HCl in aqua dest, and the absorbance was determined spectrophotometrically at 620 nm with a microplate reader. A standard curve with recombinant mouse TNF-α was included with each assay. TNF units are the reciprocal of a supernatant causing 50% lysis of L929 cells.

Statistical Analysis. Data were analyzed statistically with Student's *t* test (unpaired). Differences between samples were considered significant when p was less than 0.05.

RESULTS

In Vitro Proliferation of Rat Liver Macrophages. Rat liver macrophages (4×10^4/well) were cultured in 96-well flat-bottomed tissue culture plates in the absence or presence of a number of hemopoietic growth factors for 6 days and examined for [^{3}H]dThd incorporation. The results are shown in Table 1. Liver macrophages, incubated with medium or various concentrations of recombinant human IL-2 (1 to 500 U/ml), recombinant murine IL-3 (1 to 250 U/ml) or recombinant human IL-6 (1 to 1,000 U/ml), did not show significant levels of [^{3}H]dThd incorporation. However, when liver macrophages were incubated with recombinant murine GM-CSF (50 U/ml) or CSF-1 (20% L929-CM), high levels of [^{3}H]dThd incorporation were observed. The increase in [^{3}H]dThd incorporation with GM-CSF and CSF-1 was dose dependent (Fig. 1a and b). Maximum incorporation in the presence of GM-CSF was seen at 50 U/ml; higher concentrations of GM-CSF yielded less incorporation of [^{3}H]dThd. For CSF-1, a

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gradual increase in [^{3}H]dThd incorporation was seen with increasing concentrations; no maximum was observed in the concentration range used in this study (1% to 50% L929-CM). Experiments were also performed with combinations of IL-3 (250 U/ml) and IL-6 because it has been shown in hemopoietic systems that in the presence of IL-3, IL-6 causes colony development and cell maturation, which it is incapable of producing by itself. Liver macrophages incubated with IL-3 and IL-6 did not show significant induction of [^{3}H]dThd incorporation (data not shown). Furthermore, combinations of IL-3 and IL-6 with CSF-1 or GM-CSF did not result in synergism of proliferative activity (data not shown).

Kinetic studies using a pulse-labeling technique showed that, after a lag phase of 1 day, the macrophages responded with a strong increase in [^{3}H]dThd incorporation until a maximum was reached on day 5, both for CSF-1 (20% L929-CM) and GM-CSF (50 U/ml) (Fig. 2). Microscopically, an increase in the number of macrophages was observed, ultimately resulting in confluent monolayers of macrophages (Fig. 3). Continuous presence of CSF-1 and GM-CSF during culture was necessary to maintain macrophage proliferation because removal of the growth factors immediately arrested [^{3}H]dThd incorporation (data not shown).

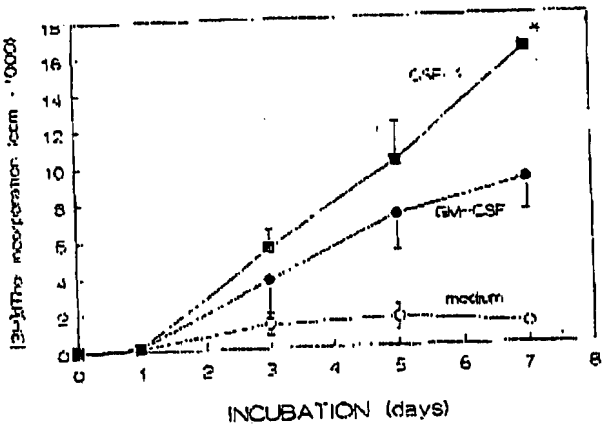

FIG. 2. Effect of incubation time on the incorporation of [^{3}H]dThd by rat liver macrophages in the absence or presence of CSF-1 (20% L929-CM) or GM-CSF (50 U/ml). Incorporation of [^{3}H]dThd was assayed as described in Materials and Methods after different time periods of incubation. Data are expressed as mean counts per minute ± S.E.M. of three individual experiments performed in triplicate. *Significant difference ($p < 0.05$) between CSF-1 and GM-CSF.

Characterization and Identification of Proliferating Cells. To characterize and identify the cells obtained after proliferation in the presence of GM-CSF and CSF-1, we performed immunohistochemical staining with specific monoclonal antibodies. Monoclonal antibody ED1 was used as a general marker for macrophages, since it recognizes the majority of macrophages of the mononuclear phagocyte system (27). Monoclonal antibody ED2, which recognizes a differentiation antigen on resident macrophages which is not expressed on monocytes (27), was used as a marker for the more differentiated resident macrophages. Monoclonal antibody Mobu-1 recognizes BrdUrd incorporated into the DNA of proliferating cells and was therefore used as a marker for proliferating cells (28).

Macrophages were incubated with or without CSF-1 (20% L929-CM) or GM-CSF (50 U/ml) for 3 and 5 days and pulsed with BrdUrd for the last 24 hr. The proportion of liver macrophages labeled with BrdUrd increased from 11% in the absence of growth factor to 47.6% and 40.7% after incubation for 5 days with CSF-1 and GM-CSF, respectively (Table 2). The percentage of BrdUrd in control groups is higher than expected. This may be a result of CSF-1 production by the isolated macrophages, which may be slightly activated as a result of the isolation procedure. The presence of CSF-1–producing fibroblasts as contaminating cells is rather unlikely; no fibroblasts were observed after several days of culture. After 5 days of culture, most of the cells grown in the presence of CSF-1 or GM-CSF had identical characteristic structure on cytospots: large cells with an indented excentric nucleus and abundant, highly vacuolated cytoplasm and a small percentage of cells containing multiple nuclei. Occasionally, mitotic figures were observed after staining with May-Grunwald-Giemsa (Fig. 4a). More than 95% of all cells stained heavily for ED1 (Fig. 4b), and more than 80% of the cells also stained for ED2, with variable intensity (Figure 4c). Macrophage proliferation is evidenced by incorporation of BrdUrd in DNA of ED1-positive cells (Fig. 4b, *arrow*). To determine whether the proliferated macrophages were phagocytic, we added latex particles to the cells. Nearly all cells conducted phagocytosis of latex particles (Fig. 4d). These results rule out the possibility that small populations of contaminating cells—including lymphocytes, endothelial cells and fibroblasts—were actively proliferating in our culture system and possibly influencing the data on [^{3}H]dThd incorporation.

Cytotoxic Properties of Proliferating Cells. In these experiments, we determined different cytotoxic parameters of the proliferating macrophages. For this purpose, macrophages were cultured in the presence of GM-CSF (50 U/ml) or CSF-1 (20% L929-CM). After 6 days, cells were detached from the culture flasks and seeded in 96-well plates (2×10^5/well) together with prelabeled C26 colon adenocarcinoma tumor cells and various activation stimuli. After a 48-hr coculture period macrophage-mediated cytotoxicity was determined. In a parallel experiment, we examined the ability of the proliferated macrophages to secrete TNF-α and NO in response to activation stimuli because these agents are thought to play an important role in cell killing by activated macrophages (32). Liver macrophages incubated for 6 days with GM-CSF or CSF-1 were not cytotoxic toward C26 adenocarcinoma tumor cells when incubated in culture medium without activation stimuli (Table 3). At the same time, no production of NO or TNF could be detected, indicating that the proliferating macrophages were not in an activated state. However, when macrophages were incubated with MDP or LPS,

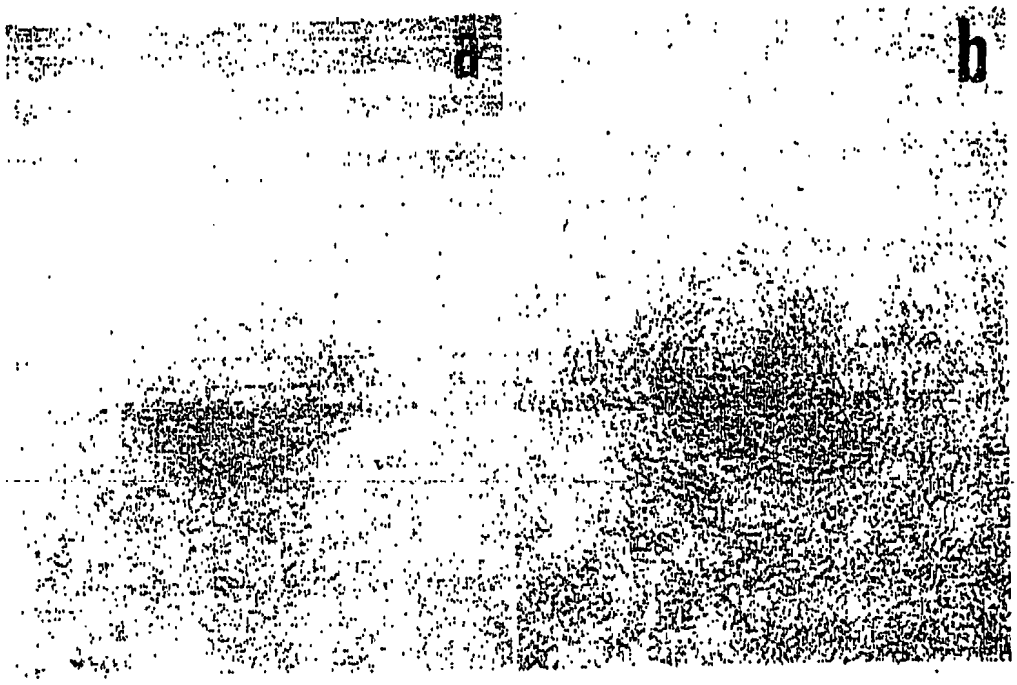

FIG. 3. Increase in cell number of rat liver macrophages incubated with CSF-1 for 6 days. Phase-contrast micrographs (×40) of cells in culture with (a) medium or (b) CSF-1 (20% L929-CM).

TABLE 2. Proportion of liver macrophages labeled with BrdUrd

Culture condition	Labeled macrophages (% total macrophages)	
	Day 3	Day 6
Medium	11.1 ± 5.9[a]	10.9 ± 6.0
CSF-1 (20% L929-CM)	40.7 ± 8.3	47.6 ± 10.9
GM-CSF (50 U/ml)	23.9 ± 3.0	40.7 ± 7.4

Liver macrophages (4×10^4/well) were incubated with or without indicated growth factors for 3 and 5 days and labeled with BrdUrd (10 μmol/L) for the last 24 hr. Macrophages with labeled nuclei were detected immunohistochemically as described in Materials and Methods.
[a]Data expressed as mean ± S.D. of three experiments.

strong induction of tumoricidal activity was observed, together with an increase in the amount of secreted NO and TNF-α. These results indicate that liver macrophages, after proliferation under control of GM-CSF or CSF-1, still have the ability to be activated to display cytotoxic properties. Incubation of the macrophages with CSF-1 or GM-CSF did not result in activation to tumor cytotoxicity or NO/TNF-α secretion.

Effects of CSF-1 and GM-CSF on Proliferation of Subfractions of Liver Macrophages Our findings raised the question of whether all or only a fraction of the liver macrophages are responsive to CSF-1 or GM-CSF. To investigate this, we separated the macrophage population into four subfractions according to cell size, which were tested for proliferative capacity during incubation with CSF-1 (20% L929-CM) or GM-CSF (50 U/ml). The results indicate that all four fractions were responsive to CSF-1 or GM-CSF but that subfractions containing the medium and large macrophages (fractions C and D) had greater potential to proliferate in response to either factor than did the small macrophages (fractions A and B) (Fig. 5). After 6 days in culture in the presence of either growth factor, cells from all subfractions displayed structure similar to that described above for the whole liver macrophage population (not shown).

DISCUSSION

Liver macrophages, as well as other tissue macrophages, are often considered terminally differentiated cells, derived from blood monocytes, with little if any capacity to proliferate (7). However, several studies suggest that liver macrophages can proliferate locally and survive by self-renewal (6, 10). Mouse liver macrophages have been shown to proliferate *in vitro* in the presence of glucan (33) or L929-CM (34, 35). Our results, obtained with rat liver macrophages, are consistent with these data. In this study, we extend these observations by demonstrating that not only L929-CM

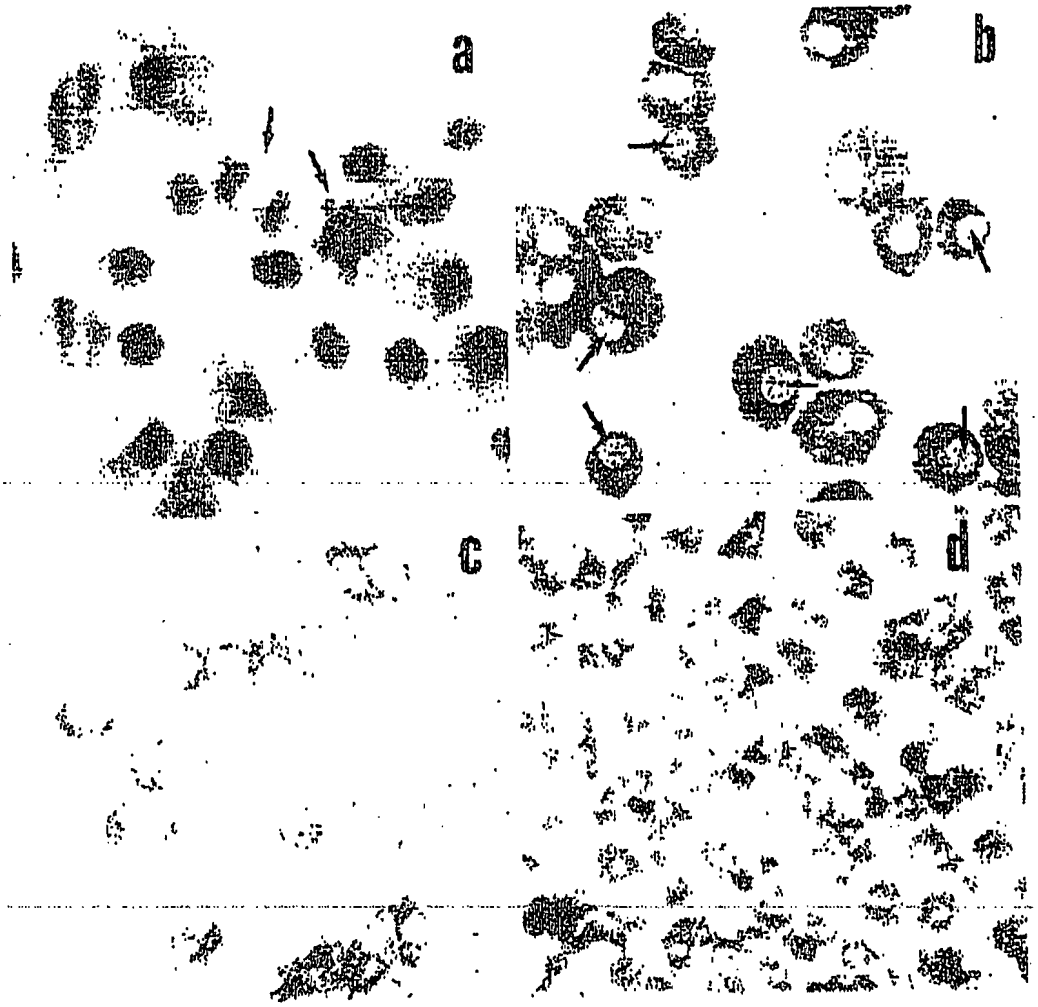

FIG. 4. Characterization of rat liver macrophages derived after 6 days of incubation with CSF-1 (20% L929-CM). Cytospots of macrophages were stained with (a) May-Grunwald-Giemsa (*arrow* indicates mitotic figure) (×800), (b) monoclonal antibody ED1 (cytoplasm) and BrdUrd (*arrow* denotes nucleus) (×510) and (c) monoclonal antibody ED2 (×510). (d) Phase-contrast micrograph of cultured cells after phagocytosis of latex particles (×150). Latex beads were added to the medium 2 hr before the end of incubation.

TABLE 1. Cytotoxic properties of rat liver macrophages derived after incubation for 6 days with CSF-1 or GM-CSF

Culture condition	Cytotoxicity (%)		NO secretion (μmol/L nitrite)		TNF-α secretion (U/10⁶ cells)	
	CSF-1	GM-CSF	CSF-1	GM-CSF	CSF-1	GM-CSF
Medium	13.5[a]	14.1	3.1	2.7	0	0
MDP (100 μg/ml)	27.2	32.5[b]	24.1[b]	41.0[b]	48.0[b]	19.0
LPS (100 ng/ml)	57.5[b]	62.5[b]	49.9[b]	55.0[b]	49.5[b]	38.0[b]
CSF-1 (20% L929-CM)	10.9	11.5	3.4	2.0	0	0
GM-CSF (50 U/ml)	11.9	16.2	3.7	2.3	0	0

Rat liver macrophages were grown in culture flasks in the presence of CSF-1 (20% L929-CM) or GM-CSF (50 U/ml). After 6 days, cells were washed and detached from the culture flasks. A total of 2×10^5 cells/well were seeded onto 96-well plates and incubated in medium with or without the indicated immunomodulators. The percentage of cytotoxicity against C-26 adenocarcinoma cells, in combination with the release of nitrite (as a measure of NO) and TNF-α into the medium, was determined as described in Materials and Methods.

[a] Data expressed as the mean of three independent experiments.

[b] $p < 0.01$ vs. medium.

[c] $p < 0.05$ vs. medium.

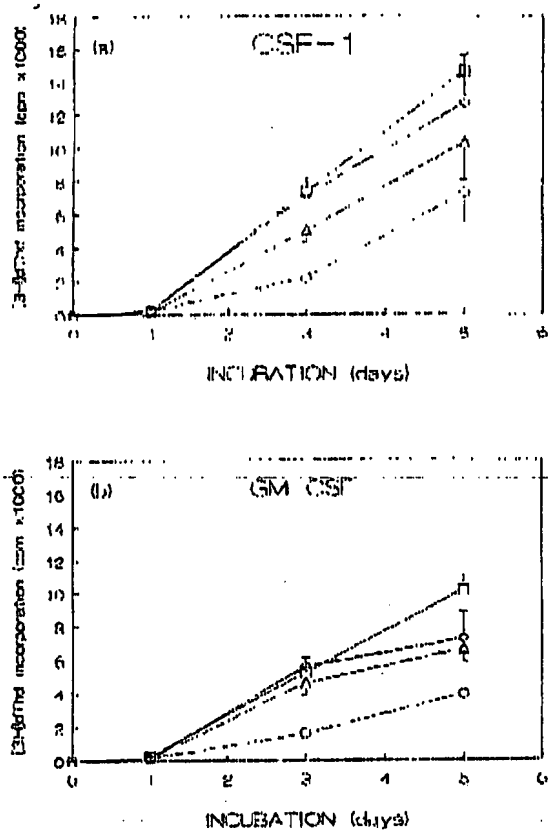

FIG. 6. Effects of (a) CSF-1 and (b) GM-CSF on proliferation of subfractions of rat liver macrophages. Macrophages (4×10^4/well) of fraction A (○), fraction B (△), fraction C (◇) and fraction D (□) were incubated with CSF-1 (20% L929-CM) or GM-CSF (50 U/ml) for different time periods. Data are obtained and expressed as described in legend of Figure 2.

(M-CSF or CSF-1) but also GM-CSF, which bears no structural similarity to CSF-1, stimulates the proliferation of rat liver macrophages *in vitro*. These results are comparable to earlier reports on macrophages isolated from other sources: Alveolar macrophages (17, 36), peritoneal exudate macrophages (37), blood monocytes (19) and bone marrow–derived macrophages (19, 22) have all been shown to proliferate *in vitro* on incubation with CSF-1 or GM-CSF. In contrast, IL-2, IL-3 and IL-6, which induce proliferation of macrophages or their precursors in bone marrow and various other organs, did not stimulate liver macrophage proliferation *in vitro*. Additionally, combination of IL-3 and IL-6, which has been shown in hemopoietic systems to cause colony development and cell maturation did not result in induction of proliferation. Because it has been shown that there is a hierarchy of hemopoietic growth factors acting during different stages of macrophage differentiation (14, 22), the results described in this study suggest that liver macrophages, capable of proliferating in these experiments, represent cell types that are in a differentiated state and differ from immature macrophage precursor cells, which were described previously (21). This is in line with our observation that rat liver macrophages are heterogeneous in their response to CSF-1 and GM-CSF to proliferate in vitro (i.e., large macrophages have higher proliferative capacity than small macrophages in response to either growth factor). It has been speculated that difference in cell size, within a given macrophage population, may be a reflection of difference in maturation level. In addition, it has been observed that the number of M-CSF receptors increases with cell maturation (11). This would be compatible with our observations on heterogeneity in growth factor response and suggests that the increase in [^{3}H]dThd incorporation is due to proliferation of the most differentiated liver macrophages. Recently Inamura et al. (38) described comparable results with isolated human monocytes. Medium and large monocytes displayed stronger proliferative responses to GM-CSF than did small monocytes. In an attempt to identify the cells capable of proliferation, we tried to determine the number of cells capable of forming colonies *in vitro*. However, in our system, no colony formation could be observed with CSF-1 or GM-CSF in culture plates (limiting dilution), soft agar or methyl cellulose. We cannot explain this phenomenon, but it is possible that the lack of colony formation is due to the differentiation state of the liver macrophages.

After culture for 6 days in the presence of CSF-1 or GM-CSF, all cells in the four macrophage subfractions display similar structure; they appear as large cells with indented nuclei, strongly staining for ED1. This suggests that the macrophages not only proliferate but also differentiate. Furthermore, proliferating liver macrophages show functional properties similar to those of freshly isolated resident liver macrophages. After 6 days of incubation with CSF-1 or GM-CSF, more than 95% of all cells were able to carry out phagocytosis of latex particles. Additionally, high levels of cytotoxicity were established with MDP and LPS, accompanied by induction of secretion of NO and TNF-α. Daemen et al. (39) have shown that liver macrophages cultured in the absence of CSF-1 or GM-CSF gradually lose their potential to become activated to tumor cytotoxicity by MDP or LPS, ultimately (after 3 or 4 days) resulting in a fully nonresponsive population.

In conclusion, our results indicate that CSF-1 and GM-CSF may play important roles among the regulatory factors that support local proliferation of macrophages in the liver. Because liver macrophages reside in close proximity to hepatocytes, it is of interest that these cells are recently shown to be capable of producing M-CSF *in vitro* (40). Whether hepatocytes also constitute a source of M-CSF *in vivo* must still be addressed. The possibility of increasing the resident liver macrophage population by way of GM-CSF or CSF-1 may be an interesting subject for further research; an increased liver macrophage population may be a physi-

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proximity to hepatocytes, it is of interest that these cells are recently shown to be capable of producing M-CSF *in vitro* (40). Whether hepatocytes also constitute a source of M-CSF *in vivo* must still be addressed. The possibility of increasing the resident liver macrophage population by way of GM-CSF or CSF-1 may be an interesting subject for further research; an increased liver macrophage population may be a physiological component of host defense against infectious and neoplastic diseases and may play a beneficial role in immunotherapeutic treatment of liver metastases. Finally, the possibility of obtaining high quantities of Kupffer cell derived macrophages can be useful for *in vitro* experiments requiring large amounts of liver macrophages.

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EXHIBIT 4

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The Immunology of Fibrogenesis in Alcoholic Liver Disease

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• ***Context.***—**Alcoholic liver disease in humans frequently leads to cirrhosis. Experimental models of hepatic fibrogenesis are available, but extrapolation of those findings to human ethanol-induced liver injury is difficult. Hepatic ethanol-induced fibrosis in humans has often been studied in relatively small patient populations. During the past decade, several animal models and human studies have attributed fibrogenesis in the liver to the role played by hepatocytes, Kupffer cells, endothelial cells, and especially stellate cells.**

Objective.—**To determine the contribution of the main liver cell types to ethanol-induced fibrogenesis. For that purpose, we studied the expression of the following immunologic parameters: smooth muscle–specific α actin (SMSA), CD68, CD34, transforming growth factor β1, intercellular adhesion molecule 1, and collagen types 1 and 3. The Dako LSAB+ kit (peroxidase method) was used.**

Design.—**We recently studied a large cohort of patients with alcoholic liver disease in France. In this cohort, we found 87 cases in which liver biopsies revealed only pericentral injury with nonpathologic portal areas. We compared cases in which the portal areas were nonpathologic with 324 patients in whom staging ranged from F0 to F3. Patients with cirrhosis (F4) were excluded from evaluation. To stage fibrosis, we used the METAVIR system. Furthermore, we selected 40 cases in which the biopsies measured at least 25 mm in length for further histochemical evaluation. Ten additional normal cases from our archives were used as controls. We divided this patient population into the following 5 groups of 10 patients each: group 1A, F0 with steatosis; group 1B, F0 without steatosis; group 2, F0 to F1, central injury; group 3, F3, fibrosis with multiple septa; and group 4, nonpathologic livers (controls).**

Results.—**Smooth muscle–specific α actin was expressed by stellate cells, pericentrally, with increasing severity and intensity in the advanced stage of fibrosis of group 3, less intense expression was noted in group 2, and expression was practically absent in group 1 and in nonpathologic controls. CD68 was the best marker for Kupffer cells and was expressed diffusely within the lobules in all groups. Its expression correlated directly with the degree of disease severity, progressing from stage I through stage III, but was absent in nonpathologic livers. CD34 was consistently expressed by endothelial cells in the periportal areas in all groups. The expression of collagen type 1 was intense in the bands of fibrosis or bridging, while type 3 expression was poor. Transforming growth factor β1 and intercellular adhesion molecule 1 were not expressed in any group.**

Conclusions.—**In this study, stellate cell activation (SMSA) was most intense pericentrally in the early stages and diffusely with progression to fibrosis and maximum intensity in stage III. Kupffer cell activation, as determined by CD68 expression, was intense and diffuse, while endothelial cells expressed CD34 periportally in a similar manner in all stages. Fibrogenesis in human ethanol injury is due to the activity of stellate cells, Kupffer cells, and to a lesser extent, to endothelial cells.**

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Cirrhosis of the liver for many years was classified based on the amount of connective tissue present and the variable size of its nodularity. Based on these parameters, the classic writers differentiated several forms of cirrhosis using names reflecting the opinion of the investigators concerning the etiology of any given case. Notable among these works were the reports of Mallory[1] and Gall.[2] Concerning the problem of alcoholic liver disease (ALD), the end stage of this condition, initially known as Laennec cirrhosis, was later renamed nutritional, micronodular, and portal cirrhosis. Mallory had already noted that several stages characterized alcoholic liver injury, but the identification of acute alcoholic hepatitis as a particularly severe form of the disease preceding the stage of cirrhosis was, surprisingly enough, not sufficiently recognized until 1961–1962 by Beckett et al.[3,4] Credit for the identification

For editorial comment, see p 1212.

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of the perivenular nature of the early injury caused by ethanol should be given to Edmondson et al.[5] Finally, an International Group in 1981 categorized the clinicopathologic manifestations of ALD,[6] allowing sequential studies that led to the realization of the dismal prognosis of ALD in its various stages.[7] However, a thorough, detailed evaluation of the various forms of early perivenular ethanol injury and fibrogenesis has not been undertaken.

Recently, we had the opportunity to study a large population of alcoholic patients in France. These patients had undergone needle liver biopsies shortly after admission and before any form of therapy was given. Clinicopathologic evaluation and the nature of the early perivenular lesions of necrosis and fibrosis were determined by histologic and immunologic means. The results of this evaluation are reported here.

MATERIALS AND METHODS

The patient population was obtained from Antoine Béclère Hospital in Clamart, France. All patients had consumed ethanol daily for at least 1 year and many of them for many years. Mean alcohol consumption in this group consisted of 50 g of ethanol daily for 1 to several years, in contrast with the US criteria (80 g/d for 1 or more years) for inclusion in the Veterans Affairs Cooperative Studies.[7] The following clinical and laboratory parameters were evaluated in the present study: age; sex; amount of ethanol consumed daily; number of years of ethanol consumption; prothrombin time (PT); serum bilirubin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), and γ-glutamyltransferase (GGT) levels on admission or at the time of the biopsy; liver span in cm; splenomegaly; ascites; and esophageal varices. Only patients who were negative for hepatitis B virus, hepatitis C virus, and human immunodeficiency virus were entered into the study.

The liver biopsies were evaluated for fibrosis following the METAVIR system,[8] with minor modifications to allow for the separation of those patients in whom the tissue revealed only perivenular (central) injury (87 cases). Staging identified the following 5 general groups: group F0, 111 patients; group F1, 122 patients; group F2, 57 patients; group F3, 34 patients; and group F4, 179 patients. Of this total number of biopsies, in 37 cases the biopsies were considered inadequate for evaluation. A biopsy was considered inadequate if no liver tissue was found in the amount minimally necessary to establish a diagnosis with morphologic certainty. Patients with cirrhosis (F4) were excluded from this study. Thus, from this group, a population of 324 alcoholic patients with early lesions (F0 to F3) and without cirrhosis was studied. An additional 87 patients whose biopsies were characterized only by pericentral injury with normal or minimal involvement of portal areas were included. Thus, the 324 patients included in this study were divided for the purposes of final evaluation into 3 categories: category 1 (F0), category 2 (central injury only), and category 3 (F3).

For the immunologic evaluation of fibrogenesis, we selected 40 cases. Ten nonpathologic livers from our archives were also investigated as normal controls. For the evaluation of fibrogenesis (after a thorough search of commercial sources), the following markers were found to best serve our purpose and were selected for investigation: Kupffer cells, CD68; Stellate (Ito) cells, smooth muscle–specific α actin (SMSA); endothelial cells, CD34; collagen, type 1 and type 3; cytokines, transforming growth factor β (TGF-β1), tumor necrosis factor α (TNF-α), and intercellular adhesion molecule 1 (ICAM-1). These markers were selected because preliminary results in our laboratory showed they were best expressed in our control tissues and worked well in paraffin-embedded tissues, as advertised by the manufacturer. Thus, a total of 50 samples were divided into 5 groups, as follows: group 1A (F0), steatosis present in mild to moderate degree; group 1B (F0), steatosis absent; group 2 (F0 to F1), central injury; group 3 (F3), fibrosis with multiple septa; and group 4, normal liver biopsies (controls).

Morphology

Histologic examination was performed on liver biopsies fixed in formalin and embedded in paraffin. All cases were routinely studied with hematoxylin-eosin, Masson trichrome stain for collagen type 1, reticulin stain in some instances, and iron stain. Immunoperoxidase for collagen type 3 was performed with a monoclonal antibody. Furthermore, to characterize the other immunologic markers, only monoclonal antibodies were used with the single exception of TGF-β, which was investigated with a polyclonal antibody from Promega Corporation (Madison, Wis). The monoclonal antibodies for CD34, CD68, SMSA, and collagen type 3 were obtained from Dako Corporation (Carpinteria, Calif). The selection of the best marker for each cell type or parameter investigated was made after preliminary studies showed the markers chosen to give the best results in our paraffin-embedded biopsies compared to the positive controls used. Skin was used for CD34. Lymph nodes and tonsils were used for CD68, coronary branches within the heart for SMSA, and cirrhotic livers for collagen types 1 and 3. Furthermore, positive controls were selected from patients with cirrhosis to test for the expression of markers such as TGF-β, SMSA, and collagen types 1 and 3. Normal livers were used as negative controls for markers such as CD68, CD34, and collagens.

Immunoperoxidase

The method in use at our laboratory was the Dako LSAB+ kit, peroxidase method. This kit contains labeled streptavidin biotin. Cut sections of liver biopsy specimens (4 μm thick) were mounted on glued slides, air-dried for 10 to 15 minutes, and fixed in cold acetone. The initial step consists of 3% hydrogen peroxide treatment for 5 minutes, followed by application of the primary antibody for 30 minutes. This step was followed by the application of the link antibody for 15 minutes, and after that, application of the streptavidin peroxidase for 15 minutes. After application of the substrate chromogen diaminobenzidine for 5 minutes, the slides were counterstained with hematoxylin for 2 to 5 minutes. The diaminobenzidine stock solution was stored in 1-μL aliquots at −80°C. The reagents were delivered to the sections with Dispenstirs (Becton Dickinson, Mountain View, Calif). Rinsing and washing with phosphate-buffered saline (×3) were done routinely for total periods of 10 minutes following each step after the cold acetone fixation.

Statistical Analyses

Descriptive statistics are expressed as mean values ± standard errors. The χ^2 or Fisher exact tests were used to compare qualitative variables. Comparisons between the different groups of individuals were performed with 2-way analysis of variance (ANOVA), and multiple comparisons used the Student-Newman-Keuls test. Relationships between parameters were evaluated with the Pearson correlation coefficient. Significance was defined as $P < .05$.

RESULTS

Morphologically, the following perivenular lesions were found in group 2: pericentral ballooning degeneration, pericentral steatosis, pericentral necrosis, pericentral fibrosis, and sclerosing hyaline necrosis (Figures 1 through 5). Pericentral ballooning was usually present alone, while frequently the other lesions appeared simultaneously.

Mean liver span was 11.6 ± 0.3 cm for group 1, 12.5 ± 0.4 cm for group 2, and 13.4 ± 0.5 cm for group 3. For this parameter, a significant difference was observed between group 1 and group 3, while group 2 did not differ significantly from either group 1 or group 3.

Clinical parameters are depicted in Tables 1 through 3. Analysis of clinical parameters, such as alcohol drinking

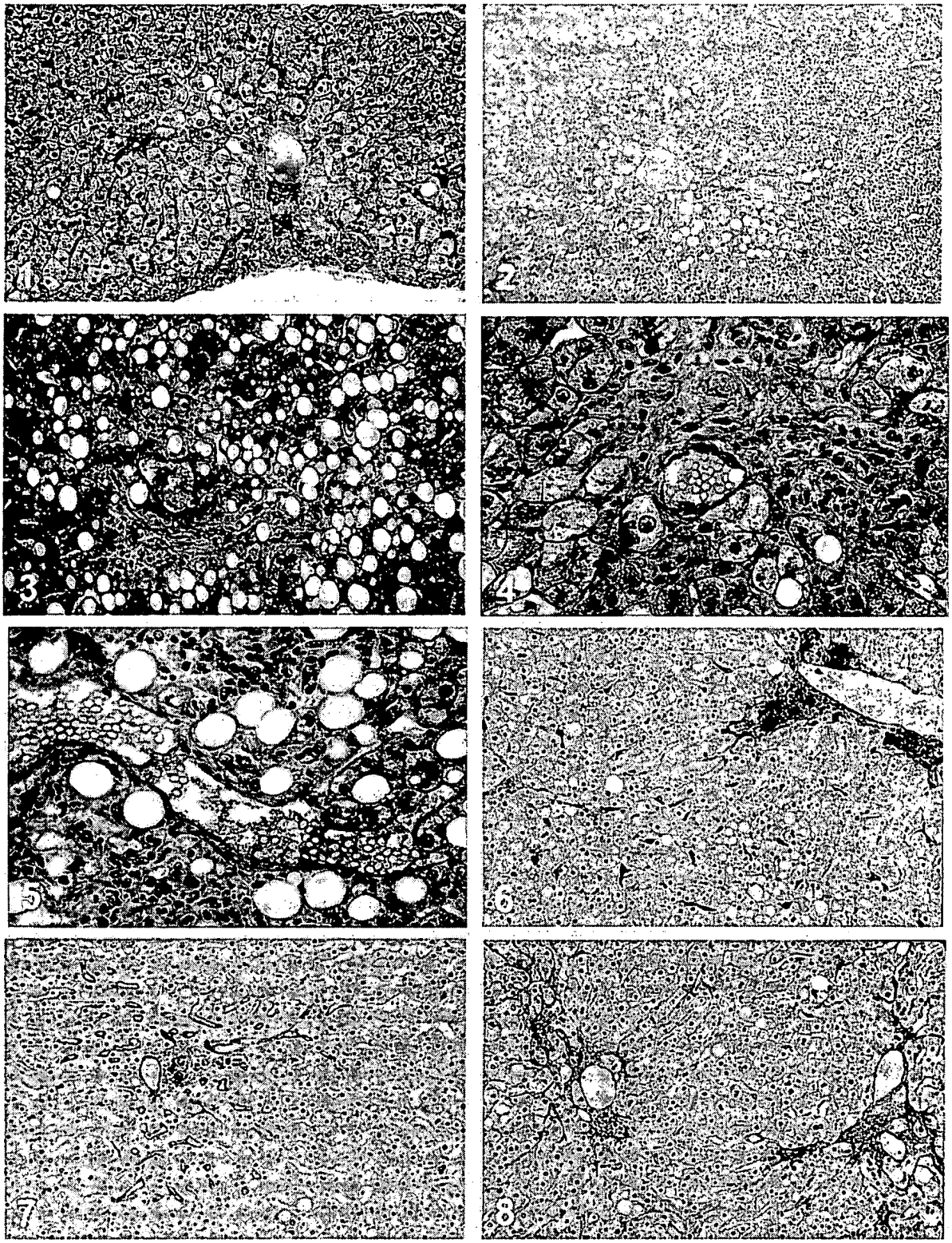

Figure 1. *Ballooning degeneration. The hepatocytes around the terminal hepatic veins (central veins) are swollen. Minimal collagen deposition is present (Masson trichrome, original magnification ×20). Collagen is normally absent in this area. Even by electron microscopy, the central veins exhibit minimal collagen, and they appear to be more like dilated sinusoids.*

Figure 2. *Pericentral steatosis. Mild collagen deposition (Masson trichrome, original magnification ×40).*

Figure 3. *Pericentral fibrosis. Central vein with collagen bands and steatosis (Masson trichrome, original magnification ×100).*

Table 1. Alcohol Drinking History*

	Age, y	Ethanol Consumption, g/d	Years of Ethanol Intake	Ratio, g/d/y
Group 1				
Men	44 ± 1 (77)	145 ± 12 (74)	16 ± 1 (73)	15 ± 2 (72)
Women	47 ± 3 (27)	111 ± 17 (26)	15 ± 3 (25)	16 ± 4 (25)
Total	**45 ± 1 (104)**	**136 ± 10 (100)**	**16 ± 1 (98)**	**16 ± 2 (97)**
Group 2				
Men	49 ± 2 (40)	136 ± 14 (39)	21 ± 2 (37)	11 ± 2 (37)
Women	53 ± 2 (20)	80 ± 14 (18)	14 ± 3 (18)	22 ± 6 (16)
Total	**51 ± 1 (60)**	**118 ± 11 (57)**	**19 ± 2 (55)**	**14 ± 3 (53)**
Group 3				
Men	49 ± 2 (21)	109 ± 9 (19)	23 ± 3 (19)	9 ± 3 (19)
Women	47 ± 4 (12)	124 ± 26 (9)	13 ± 3 (9)	14 ± 4 (8)
Total	**48 ± 2 (33)**	**110 ± 11 (28)**	**20 ± 2 (28)**	**11 ± 3 (27)**
		***P* Values**		
2-Way analysis of variance				
Group	.03†	.39	.57	.54
Gender	.36	.06	.006†	.13
Group × gender	.54	.39	.13	.35
Multiple comparisons for significant differences				
Group 1 vs group 2	.02†	. . .‡	. . .	. . .
Group 1 vs group 3	.38	. . .	. . .	. . .
Group 2 vs group 3	.21	. . .	. . .	. . .

* Values are presented as mean ± SEM of (n) subjects.
† Indicates *P* values of differences that are statistically significant.
‡ Ellipses indicate data not computed.

history, revealed statistical significance in terms of age between groups 1 and 2 and in the amount of ethanol consumption between men and women (Table 1). Other clinical data were examined by 2-way ANOVA (Table 2) and revealed statistically significant differences in PT between groups 1 and 2, groups 1 and 3, and groups 2 and 3. Total bilirubin was significantly different between groups 1 and 2 and groups 1 and 3. The difference in AST levels was statistically significant only between groups 1 and 3, while ALT did not exhibit any significant differences. Finally, GGT was significantly different between groups 1 and 2 and between groups 1 and 3.

The 2-way ANOVA was performed to detect any sex differences. A significant difference was observed between sexes; the more severe the degree of fibrosis, the lesser the duration in years of ethanol consumption by women in relation to men, that is, women ingested much less ethanol to achieve the same degree of fibrosis as the men.

The Pearson correlation coefficients (Table 3) revealed statistically significant positive correlations between age, grams per day of ethanol consumption, number of years, and the ratio of grams/day/number of years of alcohol consumption ($P < .001$). Prothrombin time correlated with total serum bilirubin ($P < .001$) and GGT levels ($P = .005$). Furthermore, serum bilirubin correlated with AST ($P = .01$) and GGT levels ($P < .001$). Finally, GGT levels correlated with PT ($P = .005$), total bilirubin ($P < .001$), AST ($P < .001$), and ALT ($P = .002$). The pairs of values with positive correlation coefficients and *P* values less than .05 tended to increase together. For the pairs with negative correlation coefficients and *P* values less than .05, one variable tended to decrease while the other increased.

←

Figure 4. *Pericentral necrosis. Ballooning of hepatocytes with moderate fibrosis ("chicken-wire") and some inflammatory cells (Masson trichrome, original magnification ×400).*

Figure 5. *Sclerosing hyaline necrosis. Fibrosis, steatosis, and acute inflammation (Masson trichrome, original magnification ×400).*

Figure 6. *CD68 expression by Kupffer cells. A central area extending into the sinusoids (CD68 immunoperoxidase, original magnification ×100).*

Figure 7. *Endothelial periportal expression. CD34 is expressed in the endothelial cells (CD34 immunoperoxidase, original magnification ×100).*

Figure 8. *Pericentral ballooning, stage F1. A liver lobule expressing smooth muscle–specific α actin (SMSA) during an early stage of ballooning with F1 (SMSA immunoperoxidase, original magnification ×100).*

Table 2. Clinical Data*

	PT	Total Bilirubin	AST	ALT	GGT
Group 1					
Men	97 ± 1 (77)	14 ± 1 (77)	36 ± 5 (77)	35 ± 5 (77)	125 ± 14 (77)
Women	95 ± 3 (27)	10 ± 1 (27)	27 ± 5 (27)	25 ± 4 (27)	112 ± 24 (27)
Total	**97 ± 1 (104)**	**13 ± 1 (104)**	**33 ± 4 (104)**	**33 ± 4 (104)**	**122 ± 12 (104)**
Group 2					
Men	95 ± 2 (40)	19 ± 2 (40)	44 ± 5 (40)	36 ± 4 (40)	303 ± 53 (40)
Women	89 ± 3 (20)	32 ± 12 (20)	42 ± 7 (20)	29 ± 4 (20)	338 ± 90 (20)
Total	**93 ± 2 (60)**	**23 ± 4 (60)**	**43 ± 4 (60)**	**34 ± 3 (60)**	**315 ± 46 (60)**
Group 3					
Men	78 ± 3 (21)	36 ± 9 (21)	42 ± 9 (21)	28 ± 2 (21)	371 ± 85 (21)
Women	85 ± 5 (12)	33 ± 12 (12)	64 ± 12 (12)	26 ± 4 (12)	313 ± 82 (12)
Total	**81 ± 3 (33)**	**35 ± 7 (33)**	**50 ± 7 (33)**	**27 ± 2 (33)**	**350 ± 61 (33)**
		***P* Values**			
2-Way analysis of variance					
Group	<.001†	<.001†	.02†	.70	<.001†
Gender	.72	.65	.54	.20	.78
Group × gender	.04†	.13	.18	.83	.73
Multiple comparisons for significant differences					
Group 1 vs group 2	.03†	.004†	.09	...‡	<.001†
Group 1 vs group 3	<.001†	<.001†	.02†	...	<.001†
Group 2 vs group 3	<.001†	.10	.24	...	.72

* Promthrombin time (PT), total bilirubin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), and γ-glutamyltransferase (GGT) levels were determined on admission or at the time of biopsy. Values are reported as mean ± SEM of (n) subjects.
† Indicates *P* values of differences that are statistically significant.
‡ Ellipses indicate data not computed.

Immunologic Findings

Our immunologic evaluation shows that, irrespective of the degree of fat present, the expression of markers of liver cell types is not modified in a relevant manner by the presence of steatosis. The best markers were CD68 for Kupffer cells, CD34 for endothelial cells, and SMSA for stellate cells. CD68 was the best marker for Kupffer cells and was expressed diffusely (Figure 6) within the lobules in all groups. Its expression was intense throughout, especially if severe necrosis (ballooning) was present. Expression was intense and correlated directly with degree of disease severity. It increased from stage F1 through F3, but was absent from control livers. CD34 was best expressed (in mild to moderate degree) within periportal endothelial cells in all groups (Figure 7). Expression of SMSA increased in intensity and correlated with degree of disease severity. Furthermore, SMSA expression was practically absent in controls and in group 1, was minimal in group 2, and was most intense in stellate cells in the advanced stage of fibrosis within group 3 (Figures 8 through 10). The SMSA enhancement with disease progression reached a peak in stage F3 and in the cirrhotic bands of the positive controls (Figure 11).

Collagen type 1 was mildly expressed pericentrally in group 1, more markedly and pericentrally expressed in group 2, and severely expressed in group 3. Collagen type 3 was absent in group 1, minimal in group 2, and intensely expressed in group 3. Collagen type 1 was intensely expressed in positive cirrhotic controls, while the expression of type 3 was less marked, although enhanced as well. Transforming growth factor β1, TNF-α, and ICAM-1 were not expressed. In conclusion, based on the intensity and periportal location of CD34 expression, the endothelial cells appear to play a role, as yet unknown, in the fibrogenesis of ALD.

Kupffer cells are activated early, diffusely, and intensely and precede the activation of stellate cells. The intensity of their activation, based on CD68 expression, suggests that they play an initial role, perhaps by secreting cytokines. This role could not be confirmed in this study because some cytokines could not be tested with monoclonal antibodies, which are not available commercially.

Stellate cells are the most intensely activated, and this activation progressively increases in severity from stage F1 to stage F3. The expression of SMSA within the collagen bands of F3 and positive cirrhotic controls suggests the existence of a continuum between early- and late-stage fibrogenesis. If this is the case, SMSA expression may be used to determine the transition between severe fibrosis and cirrhosis.

COMMENT

To our knowledge, this study examined the largest population of patients with ALD in the early stages to date.

Table 3. Clinical Correlations*

	Ethanol Consumption							
	g/d	y	g/d/y	PT	Total Bilirubin	AST	ALT	GGT
Age, y								
R	−0.291	0.584	−0.386	−0.121	0.110	−0.099	−0.179	−0.072
P	<.001†	<.001†	<.001†	.09	.12	.16	.01†	.31
n	186	181	180	197	197	197	197	197
Alcohol consumption, g/d								
R		−0.082	0.450	0.045	−0.074	−0.002	0.121	−0.092
P		.28	<.001†	.54	.32	.98	.10	.21
n		177	176	186	186	186	186	186
Alcohol consumption, y								
R			−0.586	−0.182	0.179	−0.088	−0.134	−0.039
P			<.001†	.01†	.02†	.24	.07	.60
n			180	181	181	181	181	181
Alcohol consumption, g/d/y								
R				0.032	−0.149	0.021	−0.083	−0.031
P				.67	.046†	.78	.27	.68
n				180	180	180	180	180
PT								
R					−0.569	−0.092	0.063	−0.201
P					<.001†	.20	.38	.005†
n					197	197	197	197
Total bilirubin								
R						0.180	0.068	0.345
P						.01†	.34	<.001†
n						197	197	197
AST								
R							.560	.453
P							<.001†	<.001†
n							197	197
ALT								
R								0.215
P								.002†
n								197

* PT indicates prothrombin time; AST, aspartate aminotransferase; ALT, alanine aminotransferase; and GGT, γ-glutamyltransferase. *R* is the Pearson product moment correlation coefficient, and n represents the number of subjects.
† Indicates *P* values of differences that are statistically significant.

Concerning the clinical parameters, significant differences were found between the various groups in age, PT, serum bilirubin, AST, GGT, and liver span. Abnormalities of these parameters allow the clinician to infer the early presence of liver dysfunction and to identify ethanol-induced injury. Clinically, serum bilirubin, PT, and GGT levels appear to be the best indicators to differentiate the 3 groups we identified, that is, group 1 (portal fibrosis, F0) from group 2 (central injury, F0 or F1) and group 3 (fibrosis with multiple septa, F3).

The evaluation with 2-way ANOVA and Pearson correlation coefficients revealed that other factors, such as age, grams per day of ethanol consumption, number of years, and the ratio of grams/day/number of years of alcohol consumption have significant positive correlations.

Morphologically, there seems to be a sequential set of lesions, starting with pericentral swelling of hepatocytes followed by pericentral steatosis, pericentral necrosis, pericentral fibrosis, and finally sclerosing hyaline necrosis. A combination of steatosis with other lesions, such as fibrosis or necrosis, was relatively common. The expression of these parameters in such a large population of patients allows us to conclude that central injury (F1) seems to characterize a group of patients intermediate between those with normal portal areas (F0) and those with bridging fibrosis (F3). The lesions of early pericentral ethanol-induced injury were observed in this study quite often. They are sequentially characterized here. Pericentral swelling (ballooning) is usually present alone. On the other hand, the other lesions frequently may appear simultaneously, that is, lesions such as steatosis with fibrosis, or fibrosis with necrosis. Thus, we believe that the perivenular lesions begin sequentially with ballooning degeneration followed by steatosis, necrosis, fibrosis, and finally sclerosing hyaline necrosis.

The second objective of this study deals with the evolution of fibrosis from the early stage of ALD. We found central vein fibrosis, although minimally expressed, at the swelling stage (early ballooning degeneration). Liver fibrogenesis has been a topic of considerable interest for many years. Experimental models have been developed preferentially in rats by administration of carbon tetrachloride,[8] dimethylnitrosamine,[9] and ethanol.[10] The objective of these studies was to gain insight into the complex process of human liver fibrogenesis, which represents one of the most common causes of human morbidity and mortality.

The development of liver fibrosis in humans has been

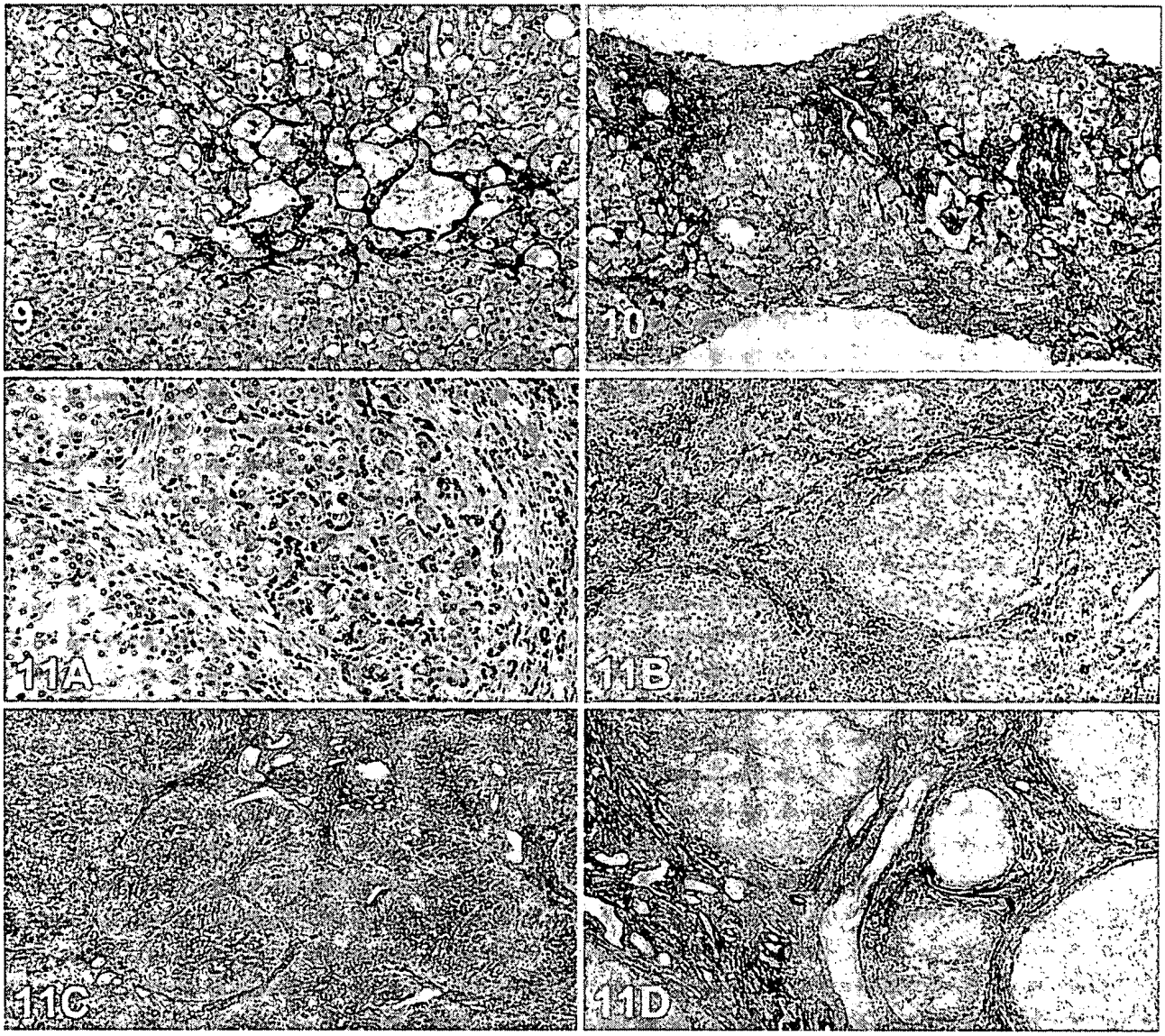

Figure 9. *Central injury, group 2, stage F2. Expression of smooth muscle–specific α actin (SMSA) pericentrally with ballooning and collapse around a central vein, stage F2 (SMSA immunoperoxidase, original magnification ×400).*

Figure 10. *Smooth muscle–specific α actin (SMSA), stage F3. A liver needle biopsy, stage F3. Porto-portal bridging. Some morphologists may be tempted to call this stage cirrhosis; however, there are no regenerative nodules and the central veins are patent (SMSA immunoperoxidase, original magnification ×20).*

Figure 11. *Positive controls in cirrhosis showing expression of 4 markers: transforming growth factor β (TGF-β1) (A), smooth muscle–specific α actin (SMSA) (B), collagen type 3 (C), and collagen type 1 (Masson trichrome) (D) (immunoperoxidase for TGF-β1, SMSA, and collagen type 3, original magnifications ×100 [A], ×40 [B], ×20 [C], and ×40 [D]). The higher magnification of panel A reveals TGF-β1 expression within a regenerative nodule by the sinusoid-lining cells.*

attributed over the years to production of hepatic substances by the various cellular components of the liver under the influence of multiple noxious agents, including ethanol. Emphasis currently has been placed either on the role of central hypoxia[11] or on the release of cytokines by sinusoid-lining cells.[12] During endotoxemia, cytokines are known to undergo alterations by increasing intestinal absorption of lipopolysaccharide and lipopolysaccharide-binding protein.

This is associated, especially in Kupffer cells, with increased secretion of TNF-α, TGF-β1, interleukin (IL)-1, IL-8, reactive oxygen species, and nitric oxide. Historically, the first cellular element suspected of playing a role in fibrogenesis was the hepatocyte,[13] followed by the Kupffer cell.[12] More recently, studies of liver fibrogenesis have shifted interest to the role played by the stellate cell.[14–16] The interest in the role of Kupffer cells in endotoxemia and related states is understandable and significant with respect to liver fibrogenesis. In the present study on human ALD, the expression of CD68 by Kupffer cells parallels the expression of SMSA by stellate cells in intensity. We could not test for the presence of TNF-α, IL-1, and IL-

8 because our material had been treated with formalin and embedded in paraffin for some time. Under these circumstances (ie, formalin-fixed and paraffin-embedded tissues), monoclonal antibodies for these cytokines are not available.

Activation of fibrogenesis has been attributed to several factors, including increased intestinal permeability by ethanol, increased absorption of intestinal endotoxin, activation of Kupffer cells, transformation of stellate cells into myofibroblasts, and finally stimulation of endothelial cells. Stellate cell activation appears to be preceded by Kupffer cell production of stimulatory factors, such as TGF-β,[12] or unknown substances produced by hepatocytes after injury.[14] Factors involved in Kupffer cell activation include, among others,[11] TNF-α and TGF-β. Northern blot studies have shown production of TGF-β1 messenger RNA (mRNA) by Kupffer cells isolated from ethanol-treated rats. Furthermore, monocytes activated by intestinal lipopolysaccharide have a similar effect. This results in stimulation of stellate cells (lipocytes) with expression of collagen genes, especially collagen type 1.[17]

The bile duct ligation model yields unusually elevated levels of collagen type 1 expression by stellate cells, while the CCl_4 model shows enhanced expression (14-fold) of collagen type 1 by endothelial cells and a 43-fold increase by lipocytes (Ito cells). The mRNA for type 3 collagen in normal rat liver has been reported to be equally increased in endothelial cells and lipocytes. This mRNA elevated in these experimental models is most dramatically expressed by stellate fat-storing Ito cells.

In our study, SMSA, the marker for stellate cells, appears to be the most reliable indicator of progression of human fibrosis in ALD. Expression of SMSA is enhanced with disease progression, reaching a peak in F3, and is seen very prominently in the positive control cirrhotic bands.

CD68 is the best marker for Kupffer cells, is expressed diffusely within the lobules in all groups, increases from stage F1 through stage F3, and is absent in normal livers. This pattern correlates directly with the clinical degree of disease severity. CD34 was expressed in this study in mild to moderate degree within periportal endothelial cells in all groups.

The bile duct ligation model[17] showed that in normal animals the endothelial cell is an important producer of collagen types 1 and 4, either higher than or similar to that of the stellate cell. We tested only for collagen types 1 and 3, and we used human livers. We also used a different immunologic marker. Thus, it is difficult to explain this difference. However, based on our information using CD34 expression by endothelial cells, the results do support a lesser role for these cells in the fibrogenesis of human ALD.

The expression of mRNA for a given collagen type experimentally does not justify a direct extrapolation to the human condition. Several studies in human alcoholics[16–20] showed no distinctive clinical features. However, in one of these studies,[20] 11 patients exhibited perivenular fibrosis very prominently, and the most characteristic features were myofibroblast proliferation and collagen deposition around the terminal hepatic venules. Of the 21 venules examined in the study, myofibroblasts represented almost half of the cellular population combined with fibroblasts, while fat-storing stellate cells as such were not recognized in the connective tissue surrounding the terminal hepatic venules. Furthermore, it was postulated that fat-storing cells, fibroblasts, and myofibroblasts belonged to the same family. In addition, it was stated that a transition occurred from Ito fat-storing cells to myofibroblasts. Three of the studies[18,20,21] concluded that perivenular fibrosis was a precursor of more advanced forms of ALD, especially cirrhosis, if the patients continued their alcohol intake. Only one study[19] concluded the opposite. Perivenular fibrosis in the alcohol-fed baboon model,[20] as well as in the French model,[22] confirmed the presence of myofibroblasts and stellate cells in the perivenular area and strongly suggested that they are the cellular elements responsible for the generation of collagen.

Our study was based on a much larger number of patients, and numerous terminal hepatic veins were available for examination. Our findings support the hypothesis that perivenular injury (swelling, steatosis, fibrosis, and sclerosis) represents earlier stages of ALD from which, unless a patient stops alcohol consumption, they most likely progress to cirrhosis.

In this study, SMSA expression increased progressively in intensity and correlated with the degree of severity of fibrosis, reaching the highest level in cirrhotic bands. Expression of SMSA was absent in normal controls but was very intensely expressed in stage F3. Thus, the increased expression of SMSA within the collagen bands of F3 and cirrhotic patients suggests the existence of a continuum between severe fibrosis and end-stage cirrhosis (F4). Finally, SMSA expression may be useful to determine the transition between severe fibrosis and cirrhosis. The earlier literature cited and our findings in this large population of alcoholics support the idea that a sequence of lesions, starting with pericentral ballooning and steatosis, progresses eventually in many patients to end-stage liver disease.

The expression of collagen type 1 was absent in normal controls, but was very intense in F3 and in positive controls with cirrhosis. Collagen type 3 was progressively expressed as disease severity increased, but with a lesser intensity in the various groups.

Expression of TGF-β1 has been reported to increase following liver injury in several animal models. Furthermore, TGF-β suppresses the proliferation of hepatocytes and other epithelial elements and induces active proliferation of hepatic stellate cells. Because previous studies with the French-Tsukamoto[11,22] rat model revealed concentrations of Ito cells in the centrilobular areas and enhanced collagen production, it has been easy to assume that this phenomenon applies to most human liver diseases, including ethanol-induced fibrogenesis. Thus, it has been stated that TGF-β1 is a major factor stimulating stellate cell fibrogenic activity (enhanced collagen production), predominantly expressed in centrilobular areas, and finally that it correlates with enhanced expression of SMSA.

We were unable to confirm this correlation in our patients. The reason for this discrepancy may be related to 3 different factors: (*a*) we studied formalin-fixed, paraffin-embedded human livers from ALD patients, while in most experimental studies dealing with rat models sampling took place shortly after sacrifice; (*b*) we studied tissues already processed and stored for quite some time, while animal studies usually used fresh tissues; and (*c*) monoclonal antibodies were not available for all markers of our human studies. However, it must be noted that TGF-β1 was strikingly expressed in our positive controls

(human cirrhotic livers), which were processed in a fashion similar to the precirrhotic cases under investigation. We are unable to explain this discrepancy. This matter has been examined recently by Friedman[23] at the molecular level.

According to relatively recent publications,[24–26] it must be recognized that TGF-β does not act alone to cause liver fibrogenesis. Other factors seem to participate, and an important one seems to be hepatocyte growth factor, which appears to have effects just opposite to those of TGF-β, such as potent proliferative activity on hepatocytes. In experimental models, hepatocyte growth factor has been shown to suppress the fibrotic response and stimulate hepatocyte proliferation.[24] In vitro studies suggest that hepatocyte growth factor functions predominantly to block the effects of TGF-β and that fibrogenesis in the liver may be the result of a delicate equilibrium between these 2 factors.

Finally, another body of literature[27,28] supports the idea that the major effector cell involved in fibrogenesis is the stellate cell, following its activation by factors released in response to injury. This response is associated with increased expression of smooth muscle proteins and increased production of extracellular matrix proteins. Stellate cells exposed in culture to vasoactive substances, such as endothelin-1 and angiotensin II, respond with enhanced production of SMSA and extracellular matrix proteins. In this study, we confirmed the enhanced production of SMSA by the stellate cells.

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EXHIBIT 5

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Microscopy Research and Technique, 1997, 39:350-64

MICROSCOPY RESEARCH AND TECHNIQUE 39:350–364 (1997)

Development, Differentiation, and Maturation of Kupffer Cells

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KEY WORDS macrophages; monocytes; M-CSF; immunophenotype; ontogeny

ABSTRACT Primitive macrophages first develop in the murine and human yolk sac and then differentiate into fetal macrophages. Primitive or fetal macrophages enter the blood stream and migrate into the fetal liver. Fetal macrophages possess a high proliferative capacity and express antigens and peroxidase activity of resident macrophages with the progress of gestation: they become mature and then transform into Kupffer cells. In contrast, myelopoiesis and monocytopoiesis are not active in yolk sac hematopoiesis and in the early stages of hepatic hematopoiesis. Precursor cells of primitive or fetal macrophages exist and granulocyte/macrophage colony-forming cells develop in the yolk sac and in the early stages of fetal liver development, whereas macrophage colony-forming cells emerge and increase later in fetal liver development. In vitro, similar colonies were formed from each fetal hematopoietic cell in the presence of different macrophage growth factors. During culturing of the yolk sac cells and hepatic hematopoietic cells on a monolayer of mouse stromal cell line, ST2, primitive or fetal macrophage colonies developed before the formation of monocyte colonies, suggesting the existence of a direct pathway of differentiation from primitive macrophages into fetal macrophages during ontogeny.

In severely monocytopenic mice induced by the administration of strontium-89, Kupffer cells have a proliferative capacity and are maintained by self-renewal. In macrophage colony-stimulating factor (M-CSF)-deficient (*op/op*) mice, the number of Kupffer cells is reduced, and they are characterized by immature morphology and a proliferative potential similar to that of primitive or fetal macrophages during ontogeny. Immediately after the administration of M-CSF to *op/op* mice, Kupffer cells start proliferating and become mature. This finding indicates that M-CSF plays an important role in the differentiation and proliferation of Kupffer cells. *Microsc. Res. Tech. 39:350–364, 1997.* © 1997 Wiley-Liss, Inc.

INTRODUCTION

Kupffer cells are located in the hepatic sinusoids and are involved in the metabolism of various compounds, immunological responses, and inflammatory reactions. Since the discovery of Kupffer cells by Kupffer (1876), several diverse views on their origin have been presented. He described "Sternzellen" in the liver by using a gold chloride-staining method that stained fat-storing cells (Wake et al., 1989). Kupffer also observed in the hepatic sinusoids "endothelial cells" phagocytizing India ink after its intravenous injection (Kupffer, 1898, 1899). Kiyono (1919) also identified cells in various tissues by vital staining with lithium carmine and assumed that Kupffer cells originated from the reticuloendothelia of the hepatic sinusoid. Based on the results obtained from his collaborations with Kiyono, Aschoff (1924) established the concept of the "reticuloendothelial system" (RES), a cell system composed of reticulum cells, reticuloendothelia (phagocytic endothelia), and histiocytes (macrophages). The constituent cells of the RES were defined as mesenchymal cells stained intensely by vital staining, and all cells constituting the RES were considered to have an identical origin, morphology, and function. From these constituent cells, fibroblasts were excluded because their intensity for vital staining was slight. In the RES, Kupffer cells belonged to an element of the reticuloendothelia of the liver. However, by using a combination of perfusion fixation and ultrastructural peroxidase cytochemistry, Wisse (1974a,b) clearly demonstrated that the endothelial cells and Kupffer cells belong to two different cell populations and that Kupffer cells are resident macrophages in the hepatic sinusoid. Kupffer cells are now defined as macrophages in the hepatic sinusoid with (1) brisk phagocytosis, (2) reactivity to anti-macrophage monoclonal antibodies, (3) proliferative capacity, and (4) a localization pattern of endogenous peroxidase activity as resident macrophages.

According to the concept of the mononuclear phagocyte system (MPS) proposed by Furth (1975, 1980, 1989, 1992; Furth et al., 1972), it has been concluded that blood monocytes originate from precursor cells in the bone marrow, migrate into various tissues of the body, and transform into tissue macrophages. However, macrophages in fetal tissues including the fetal liver are known to develop before the initiation of bone marrow hematopoiesis in animals. Previous studies have revealed that macrophages in the fetal liver express cytochemical and immunohistochemical characteristics of Kupffer cells in the late stages of liver development (Bankston and Pino, 1980; Delmann and

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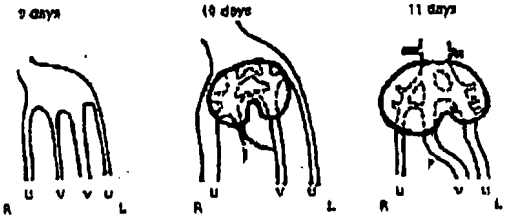

Fig. 1. Schematic representation of the development of umbilical and vitelline veins and liver anlage in the mouse embryo. U, umbilical vein; V, vitelline vein; R, right; L, left; P, portal vein. Reprinted with permission from Naito et al., 1990a.

Fahimi, 1978; Naito and Wisse, 1977; Naito et al., 1982, 1990a; Pino and Bankston, 1979). However, the precursors of these hepatic fetal macrophages and the mechanisms governing the differentiation of fetal hepatic macrophages have been poorly understood. This article describes the origin, differentiation, maturation, and proliferation of Kupffer cells not only during ontogeny but also in the adult life of mice, rats, and humans. This work is based on the results obtained from immunohistochemical, electron microscopic, and in vitro studies and on the analyses of animal models useful for investigating macrophage differentiation and kinetics.

ONTOGENY OF KUPFFER CELLS IN THE MOUSE

Macrophages are known to appear in hematopoietic tissues. The first hematopoietic organ where macrophages develop in mammals is the yolk sac (Cline and Moore, 1972; Enzan, 1986; Fukuda, 1973; Kelemen et al., 1979; Moore and Metcalf, 1970; Takahashi et al., 1989). In mouse embryos at 8.5 days of gestation, blood islands start developing in the mesenchymal layer of the yolk sac (Takahashi et al., 1989; Naito et al., 1990b). Primitive erythroblasts and immature megakaryoblasts appear among undifferentiated blood cells. At 9 days of gestation, the heart is formed and the fetal cardiovascular system is connected with the vitelline and umbilical vessels (Fig. 1). At this time, mononuclear cells are positive for murine macrophage monoclonal antibody F4/80 (Austyn and Gordon, 1981) and are immunohistochemically detected in the vascular lumen of the yolk sac (Takahashi et al., 1989). These mononuclear cells are round, possess a euchromatic nucleus with large nucleoli, a poorly developed Golgi apparatus, few cytoplasmic organelles, and abundant polyribosomes (Fig. 2). Differentiation of such immature cells into macrophages with more mature ultrastructural features is very rapid and occurs within 1 day. We designated the former immature cells as "primitive macrophages" and the latter mature cells as "fetal macrophages." Both are negative for peroxidase activity as determined by ultrastructural cytochemistry and are found in not only the vascular lumen but also in the extravascular mesenchymal layer of the yolk sac (Takahashi et al., 1989).

At fetal day 10, the fetal liver begins to form its fundamental structures beneath the venous sinus and to receive its blood supply through the umbilical veins and the left vitelline vein (Fig. 1). The umbilical veins are connected with the portal vein to form a sinusoidal network throughout the fetal liver. At this stage, hematopoiesis starts in the fetal liver and is predominantly erythroblastic. A few F4/80-positive cells exist in the mesenchymal tissue of the mouse fetus but not in the fetal liver. In the vascular endothelial channels, a number of erythroblasts and mononuclear cells are present. The mononuclear cells in the hepatic sinusoid (Fig. 3) bear ultrastructural and immunophenotypic characteristics of primitive macrophages, which appear in the yolk sac. These cells are suggested to have migrated from the yolk sac during hematopoiesis via the blood stream to colonize in the fetal liver during hepatic hematopoiesis. In fact, cord blood sampled from murine fetuses contains primitive or fetal macrophages, suggesting that the cells move through the blood stream during the fetal period (Izumi et al., 1990). The number of circulating primitive or fetal macrophages in peripheral blood decreases after fetal day 17 and disappear after fetal day 19, whereas monocytes appear in the peripheral blood after fetal day 17.

A few F4/80-positive macrophages are first detected at 11 days of gestation in the fetal liver. Their number increases with fetal age (Fig. 4). Besides primitive macrophages, macrophages exhibiting avid hemophagocytic activity are found in the sinusoidal lumen or attached to the surface of endothelial cells. At 12 days of gestation, the number of macrophages that phagocytosed various blood cells is increased and most of them are attached to the endothelial cells in the hepatic sinusoid. Such macrophages contain abundant polyribosomes, extended microvili or filopodia, and are negative for peroxidase activity by ultrastructural cytochemistry (Fig. 5). These cell characteristics are consistent with what are described as "fetal macrophages" in the yolk sac. The number of fetal macrophages in the hepatic sinusoid increase with fetal age and occasionally are found in the extrasinusoidal space. With the increase in erythropoiesis, macrophages are often found in the center of erythroblasts forming erythroblastic islands (Sasaki et al., 1993). In this fashion, fetal macrophages in the fetal liver play a scavenger function and support hematopoiesis. Such fetal macrophages begin to show peroxidase activity in the nuclear envelope and rough endoplasmic reticulum after 17 days of gestation (Fig. 6) (Deimann and Fahimi, 1978; Naito and Wisse, 1977; Naito et al., 1982, 1990b; Pino and Bankston, 1979) corresponds to that of Kupffer cells in the adult liver (Wisse, 1974a,b). Hepatic hematopoiesis becomes most prominent from 16 to 18 days of gestation, whereas it decreases in the perinatal period and disappears within a week after birth. Hemophagocytosis by macrophages becomes less prominent after 18 days of gestation. These resident macrophages rapidly increase thereafter and transform into Kupffer cells in the late stage of ontogeny and after birth. Ultrastructural and immunohistochemical features of primitive macrophages, fetal macrophages, and Kupffer cells are summarized in Table 1.

PROGENITORS OF MURINE FETAL KUPFFER CELLS

Recently, monoclonal antibodies raised against murine macrophage precursors have been produced (Leenen et al., 1990a,b). ER-MP12 antigen mainly recognizes granulocyte/macrophage colony-forming cells

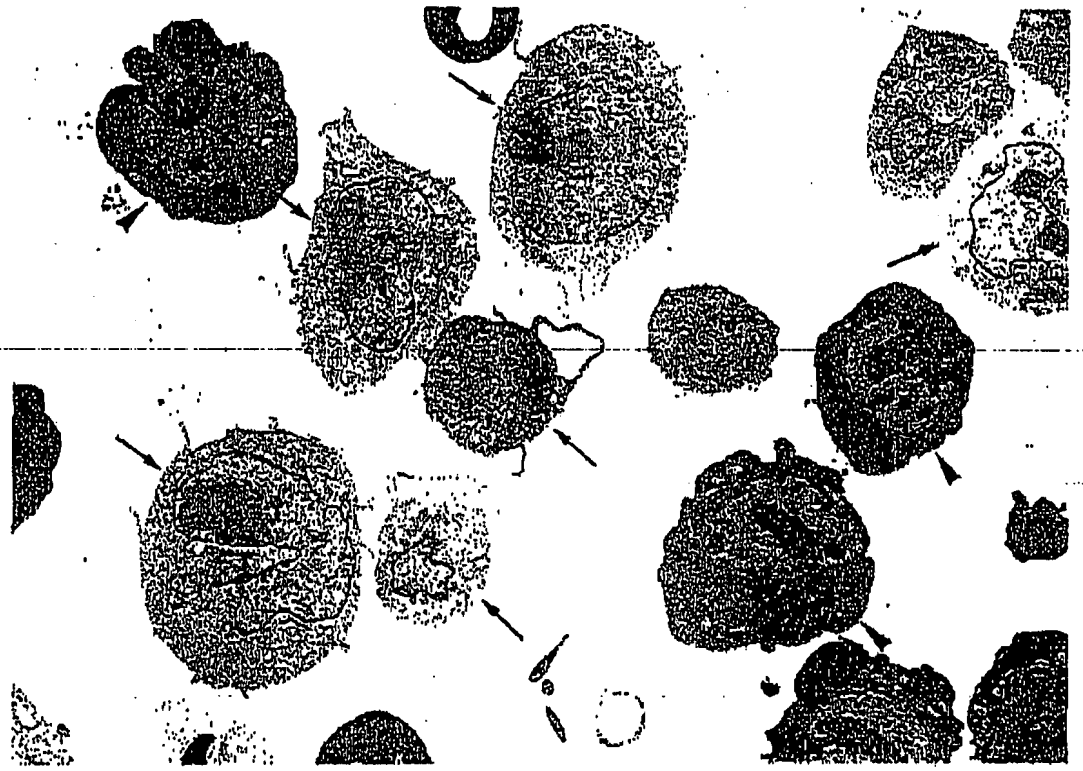

Fig. 2. Development of primitive macrophages (arrows) in the lumen of a yolk sac vessel. Arrowheads indicate erythroblasts. ×2,400. Reprinted with permission from Takahashi et al., 1989.

(GM-CFCs), and ER-MP20 antigen is expressed on macrophage colony-forming cells (M-CFCs), promonocytes, monocytes, and immature macrophages (Leenen et al., 1990a,b; Wijffels et al., 1993; Morioka et al., 1994). In the yolk sac hematopoiesis, ER-MP12-positive GM-CFCs and F4/80-positive macrophages are detected from 9 days of gestation. In the fetal liver, a few F4/80-positive macrophages and ER-MP12-positive GM-CFCs are present on fetal day 12, but ER-MP20-positive monocytic cells are few in number. In the fetal mouse liver, ER-MP12-positive cells are present throughout the fetal period, whereas ER-MP20-positive cells increase with gestational age (Fig. 7). The fact that ER-MP20-positive cells are absent in the yolk sac and during the early stages of fetal liver formation and numerous F4/80-positive macrophages exist may support the view that the F4/80-positive primitive or fetal macrophages are derived from GM-CFCs or earlier precursor cells bypassing the stage of monocytic cell series (promonocytes and monocytes) during early ontogeny. Because both ER-MP12-positive GM-CFCs and ER-MP20-positive monocytic cells are present in the late stages of hepatic hematopoiesis, both are considered to be precursors of fetal macrophages in the fetal liver during late ontogeny.

ONTOGENETIC DEVELOPMENT OF KUPFFER CELLS IN RATS

In rats, primitive or fetal macrophages develop in the yolk sac (Takahashi and Naito, 1993). They share ultrastructural features similar to those of mice, show a high proliferative capacity and immune phagocytosis via Fc receptors, and are positive for the rat macrophage monoclonal antibody RM-1 (Takeya et al., 1989). The fetal liver primordium is initially formed at 11 days of gestation, and fetal hematopoiesis starts from 12 day of gestation. In the sinusoidal lumen, there are erythroblasts, megakaryoblasts, and primitive or fetal macrophages. The processes of proliferation, differentiation, and maturation of macrophages in the fetal rat liver closely resemble those of mice, and mitotic figures of macrophages are frequently encountered. Macrophages actively phagocytize not only hematopoietic cells but also injected foreign particles or IgG-coated sheep erythrocytes (Figs. 7, 8) (Bankston and Pino, 1980; Deimann and Fahimi, 1978; Naito et al., 1982, 1986). The initial emergence of peroxidase activity in the nuclear envelope and rough endoplasmic reticulum of rat macrophages occurs from 13 to 14 days of gestation (Deimann and Fahimi, 1978; Naito and Wisse, 1977; Naito et al., 1982; Pino and Bankston, 1979) and almost all macrophages show ultrastructural features of resident macrophages at 18 days of gestation.

Our previous immunophenotypical studies have demonstrated that RM-1-positive cells first appear in the yolk sac at fetal day 9 (Takahashi and Naito, 1993). By fetal day 12, TRPM-1-, 2-, and RM-1-positive cells are observed in the fetal liver. ED1-, ED2-, and Ki-M2R-positive macrophages appear at fetal day 18. ED2

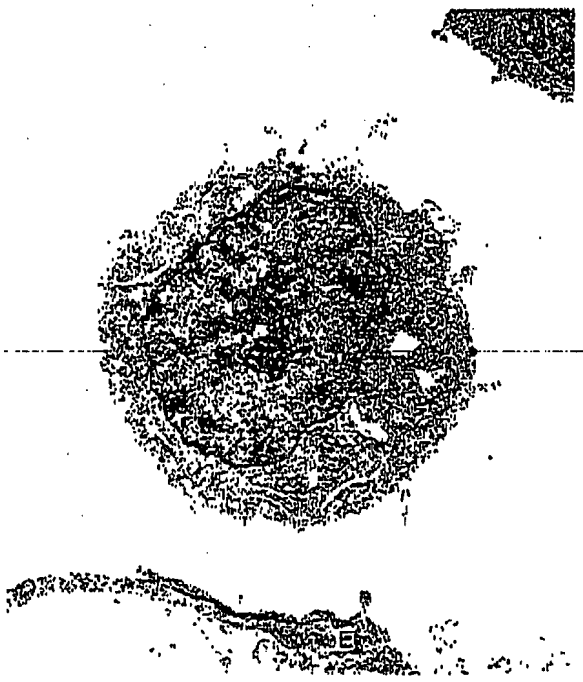

Fig. 3. Primitive macrophage in the sinusoidal lumen of the mouse fetal liver at fetal day 10. The cell shows a large nucleocytoplasmic ratio (>1), abundant polyribosomes, and short microvillous projections. E, endothelial cells. ×7,500. Reprinted with permission from Naito et al., 1990.

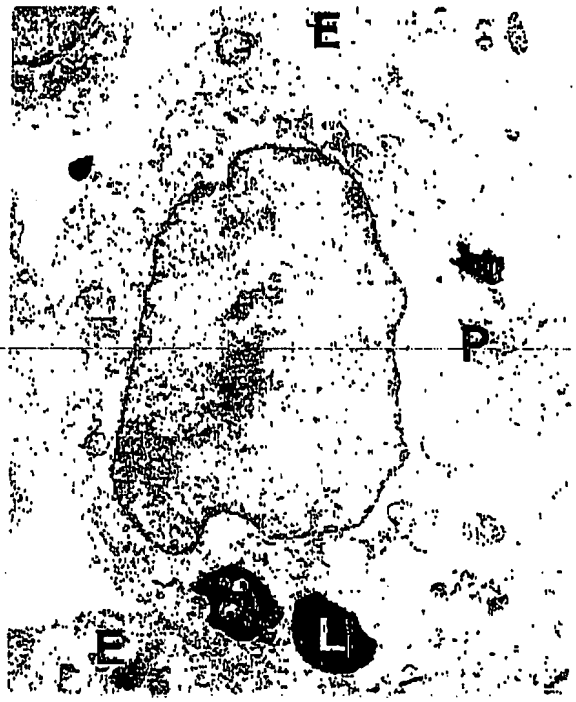

Fig. 5. Peroxidase-negative fetal macrophage in the liver at 11 days of gestation. The cell attaches to endothelial cells (E) and contains a few lipid droplets (L) and phagosomes (P). ×10,000 (Naito et al., 1990).

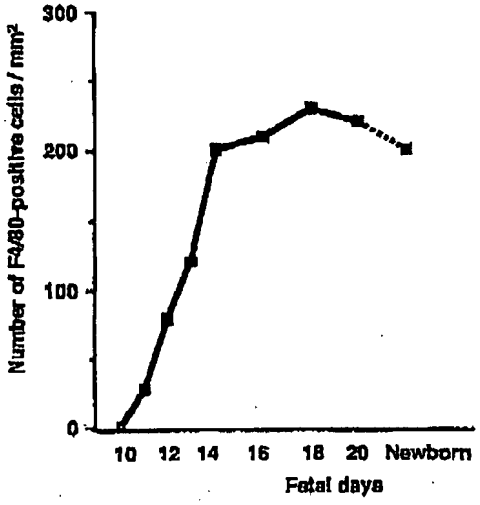

Fig. 4. Number of F4/80-positive cells in the fetal mouse liver per square millimeter.

(Dijkstra et al., 1985) and Ki-M2R (Wacker et al., 1985) recognize the distinctive antigens of resident macrophages, including Kupffer cells (Fig. 9). Because hepatic macrophages after 18 days of gestation begin to show peroxidase activity in the nuclear envelop and rough endoplasmic reticulum, ED2- and Ki-M2R-positive macrophages in the fetal liver belong to resident macrophages defined by the localization pattern of peroxidase activity. These data suggest that these macrophages are primitive or fetal macrophages originating from the yolk sac, which express further macrophage differentiation antigens and differentiate into resident macrophages. TRPM-3 and ED3 are expressed on monocyte-derived macrophages and certain restricted macrophage populations such as those in the marginal zone and omentum (Miyamura et al., 1988; Takeya et al., 1987). TRPM-3-, ED3-, and Ia-positive macrophages are considered to be monocyte derived.

PROLIFERATION OF MACROPHAGES IN THE MURINE FETAL LIVER

One of the outstanding features of macrophages in fetal tissue is their high proliferative capacity. Our previous studies with ^{3}H-thymidine autoradiography demonstrated that 40% and 60–70% of macrophages in the yolk sac at fetal day 10 are labeled in rats and mice, respectively (Takahashi et al., 1989; Takahashi and Naito, 1993). In the fetal rat liver, 63% of fetal macrophages are labeled at fetal day 14 (Fig. 10), indicating that they are a highly proliferating cell population (Naito et al., 1982). Mitotic figures of macrophages are encountered frequently (Fig. 11). In the late fetal period, macrophages in various tissues still exhibit a proliferative capacity, and their labeling rate is maintained at 6–10% (Takahashi et al., 1989; Higashi et al.,

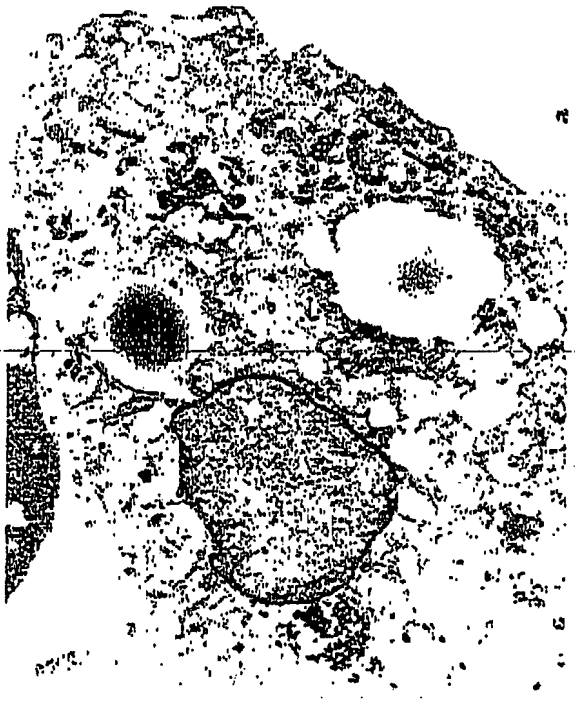

Fig. 6. Hepatic macrophage with peroxidase activity in the nuclear envelope and rough endoplasmic reticulum at 17 days of gestation. ×7,000.

1992). The proliferative potential of fetal macrophages in the liver is important for their survival in loco and for their colonization from the fetal liver to the other fetal tissues via blood stream. From this viewpoint, the fetal liver is a central organ for producing and supplying macrophages and their precursors to the other tissues.

MONOCYTE ONTOGENY IN THE MURINE FETAL LIVER

Although fetal hematopoiesis contains myeloid precursors as detected by colony-forming assays, granulopoiesis and monocytopoiesis are poorly developed during murine yolk sac hematopoiesis in vivo. Promonocytes and monocytes appear at fetal day 11 during mouse yolk sac hematopoiesis, but they are an extremely minor population. Promonocytes are ultrastructurally defined as cells with peroxidase localization in the nuclear envelope, rough endoplasmic reticulum, Golgi apparatus, and granules, but monocytes only display peroxidase activity in their granules. Compared with promonocytes or monocytes in bone marrow hematopoiesis, the number of peroxidase-positive granules in these cells are few during fetal hematopoiesis (Takahashi et al., 1989). Promonocytes in yolk sac hematopoiesis show a weaker intensity for peroxidase reaction in the nuclear envelope, rough endoplasmic reticulum, and Golgi apparatus than those in bone marrow hematopoiesis (Takahashi et al., 1989). In the fetal mouse liver, promonocytes and monocytes increase in number and show increased numbers of peroxidase-positive granules (Naito et al., 1986, 1990a). In the middle stages of hepatic hematopoiesis, their ultrastructural features resembled those seen during bone marrow hematopoiesis (Figs. 12, 13). Therefore, the development of MPS is thought to be completed by the middle stage of hepatic hematopoiesis (Naito et al., 1986, 1990a). The number of myeloid cells with peroxidase-positive granules increases with gestational age in the fetal liver, whereas immature myeloid cells are replaced by metamyelocytes or neutrophils until birth. These metamyelocytes and neutrophils also decrease in late ontogeny and disappear soon after birth. In the late fetal stage, monocytes are detected in the peripheral blood, and TRPM-3- or ED3-positive macrophages appear in tissues (Higashi et al., 1992; Izumi et al., 1990; Naito et al., 1986; Wake et al., 1989), suggesting that they migrate into fetal tissues and differentiate into macrophages.

DEVELOPMENT AND DIFFERENTIATION OF MACROPHAGES IN THE HUMAN FETAL LIVER

In humans, an endodermal cell nest develops adjacent to the vitelline vein and forms the liver primordium at 25 days of gestation (Moore, 1988), whereas the primitive sinusoidal plexus branches from the vitelline vein. Despite the absence of hematopoiesis, the hepatic sinusoids contain a few primitive erythroblasts and macrophages, suggesting that they are of yolk sac origin. Indeed, macrophages and erythroblasts are predominantly produced in the yolk sac at that stage. In agreement with previous studies (Enzan et al., 1983; Fukuda, 1974; Kelemen and Janossa, 1980), a large number of macrophages develop in the sinusoidal lumen after 30 days of gestation and are often aggregated or in clusters (Fig. 14). They possess numerous filopodia or microvilli, an abundant cytoplasm, numerous dense bodies, and exhibit prominent homophagocytosis. Most of the intrasinusoidal macrophages in the human fetal liver show a well-developed ultrastructure. However, besides the mature macrophages, there also exist in embryonic livers some morphologically immature small macrophages that react positively for some macrophage-associated antigens. Large and small macrophages have a negative peroxidase reaction in any intracellular organelle. From 40 days of gestation, a large number of hematopoietic precursor cells, predominantly of the erythroid series, abruptly proliferate mainly in the extrasinusoidal area, and macrophages are also found both in and out of the sinusoid. Although a few immature granulocytes are often present in the connective tissues of portal triads (Enzan et al., 1983; Emura et al., 1983; Fukuda, 1974), mature granulocytes or monocytes are not identified, although our recent immunohistochemical observations have demonstrated only a few CD15-positive myeloid cells (Fig. 15). Flow cytometric analysis revealed a distinct population of CD14-positive monocytic cells in the bone marrow from an 11-week-old fetus; however, hepatic hematopoietic cells lacked such a CD14-positive monocytic population. Thus, monocyte/granulocyte production is also incomplete in the early stages of human hepatic hematopoiesis (Enzan et al., 1983; Fukuda, 1974; Hasegawa and Naito, 1995), which is similar to that seen in the murine liver.

TABLE 1. Ultrastructural, cytochemical, immunoelectron microscopical, and functional features of primitive macrophages, fetal macrophages, Kupffer cells, and monocytes in mice and rats[1]

	Primitive macrophages	Fetal macrophages	Kupffer cells	Monocytes
Nucleus	Round, indented	Indented	Indented	Reniform
Nucleolus	++	+	±	±
Chromatin	Euchromatic	Heterochromatic	Heterochromatic	Heterochromatic
N/C ratio	≧1	<1	<1	<1
rER	Few	+	++	++
Golgi	Poor	+	++	+
Lysosome	-	+	++	+
Phagocytic vacuoles	-	+	++	±
Pinocytic vesicles	Few	+	++	+
Polyribosomes	++	+	-	-
Filopodia	+	+	+	-
Microvilli	±	+	++	+
Pseudopodia	-	+	+	-
Peroxidase				
Nuclear envelope	-	-	+	-
rER	-	-	+	-
Golgi	-	-	-	-
Granule	-	-	-	+
F4/80	+	+	+	+
RM-1	+	+	+	-
EA rosette formation	+	+	+	+
Immune phagocytosis	+	+	+	+
Latex phagocytosis	Slight	+	++	±

[1]N/C ratio, nucleocytoplasmic ratio; rER, rough endoplasmic reticulum; -, absent; +, present; ++, abundant; ±, occasionally present; F4/80, anti-mouse macrophage monoclonal antibody; RM-1, anti-rat macrophage monoclonal antibody.

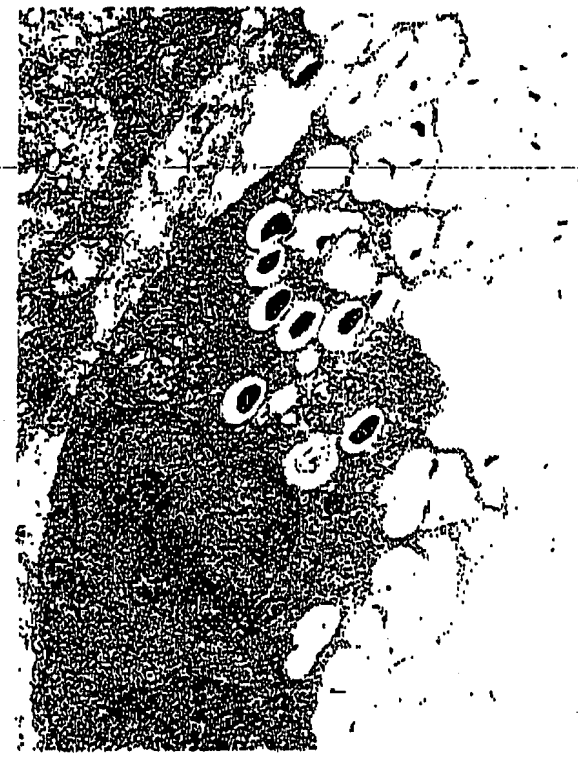

Fig. 7. Fetal rat macrophage phagocytizing injected latex particles (0.81 μm) at 18 days of gestation. ×10,000.

Fig. 8. Fetal rat macrophage phagocytizing IgG-coated sheep erythrocytes via Fc receptors. ×6,000.

Immunophenotypical heterogeneity of human fetal macrophages has been reported (Bardadin et al., 1991; Bhoopat et al., 1986; Kamps et al., 1989; Timens et al., 1990). From 30 days of gestation, almost all of the macrophages including small immature and large mature macrophages express CD68 and a human macrophage-related antigen as recognized by a macrophage monoclonal antibody HAM56. The PM-2K and CD14

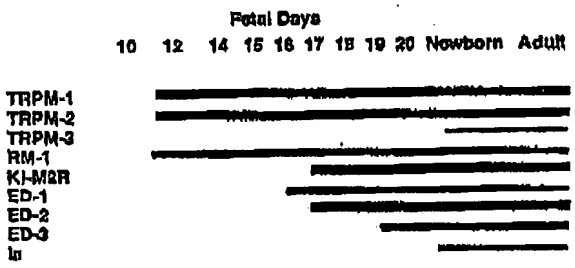

Fig. 9. Immunophenotypic expression of fetal rat macrophages in the liver.

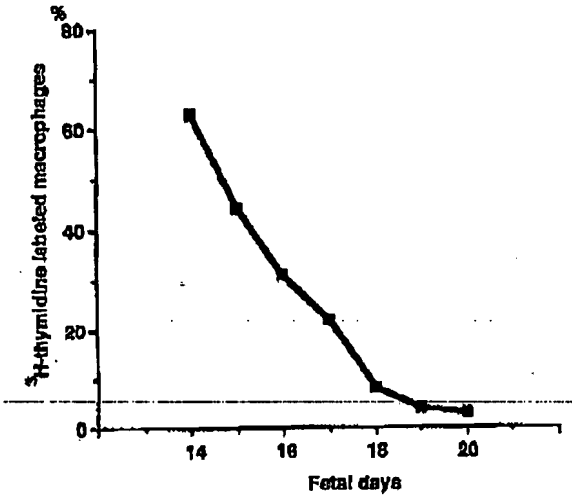

Fig. 10. Proliferation of macrophages in the fetal rat liver. The percentage of ^{3}H-thymidine-labeled EA rosette-forming cells is presented.

antibodies identify a subpopulation of mature macrophages, whereas CD14 antibodies also stain a small population of immature macrophages (Fig. 15). These observations indicate that hepatic macrophages are comprised of immunophenotypically heterogeneous macrophage populations and that the small ultrastructurally immature macrophages recognized by some macrophage monoclonal antibodies represent a human counterpart of a primitive macrophage population identified in mice and rats (Hasegawa and Naito, 1995).

FETAL MACROPHAGE DIFFERENTIATION IN VITRO

For a further analysis of the mechanism of macrophage differentiation, we conducted in vitro studies of fetal hematopoietic cells under various conditions. In cultures of cell suspensions from the fetal liver in the soft agar using I.P3-conditioned medium, granulocyte/macrophage (GM) colonies developed. The number of GM colonies in cultures of cells from the fetal liver was about 40% of those in cultures of bone marrow cells from adult mice (Naito et al., 1990a). These findings indicate that abundant macrophage precursors exist in the fetal liver (Fig. 16).

Colony-stimulating factors (CSFs) are largely responsible for defining the role of the microenvironment for macrophage phenotypes and functions (Falk and Vogel, 1990; Falk et al., 1991; Rutherford et al., 1993). In the fetal liver, hepatocytes are considered to play a major role in hematopoiesis, and CSFs are detected in the liver during the fetal development (Azoulay et al., 1987; Roth and Stanley, 1996). Recently, a hepatocyte cell line established from the murine fetal liver was shown to be capable of supporting hematopoiesis by producing M-CSF and GM-CSF (Hata et al., 1993). Thus, hepatocytes provide a microenvironment for the development, differentiation, and proliferation of macrophages in the fetal liver by producing CSFs, and macrophage CSF (M-CSF) is also known to be produced from the yolk sac (Azoulay et al., 1987) and maternal uterus (Pollard et al., 1987). A large amount of M-CSF is produced by the luminal and glandular epithelium of the uterus through pregnancy, and the concentration of M-CSF in the uterus increases by 1,000 fold (Bartocci et al., 1986). Uterine macrophages produce a variety of cytokines that are thought to immunosuppress the host response to the fetus. M-CSF and these cytokines may also act directly on macrophages in the fetus.

To examine the effect of CSFs on macrophage development and differentiation during ontogeny, various in vitro studies have been performed. In a soft agar gel culture of bone marrow cells, yolk sac cells, and fetal liver cells, macrophage colonies formed in the presence of various CSFs (Morioka et al., 1994). There are no significant differences in the expression of macrophage differentiation antigens in the colonies between the bone marrow culture and the culture of hematopoietic cells from either the yolk sac or fetal liver. The expression of macrophage precursor-cell-related antigens in cultures of bone marrow cells is mostly comparable to that of fetal hematopoietic cells. ER-MP12 antigen is expressed in a lower percentage of colonies of bone marrow cells or fetal liver hematopoietic cells cultured in the presence of CSF as compared with the ER-MP20 antigen. In contrast, the numbers of ER-MP12-positive colonies grown in the presence of CSF are much more abundant in cultures of hematopoietic cells from the yolk sac than those of bone marrow cells or hematopoietic cells from the fetal liver. It is generally accepted that M-CSF mediates the development and differentiation of M-CFCs. Based on this notion, it is difficult to explain why M-CSF-responsive ER-MP12-positive cells develop only in the yolk sac cell culture. The fact that the expression of ER-MP12 is elevated in yolk sac cell cultures supplemented with M-CSF lends support to the phenomenon that ER-MP12-positive progenitors develop abundantly in the yolk sac (Morioka et al., 1994), a location where M-CSF production is high (Azoulay et al., 1987). This phenomenon is also explained by the fact that M-CSF upregulates the differentiation of hematopoietic cells, in particular the GM-CFCs (Wiktor-Jedrzejczak et al., 1991, 1992).

The mouse bone marrow stroma cell line, ST2, is known to produce several growth factors including M-CSF (Yoshida et al., 1990). Macrophage colonies develop in cell suspension cultures of fetal livers ob-

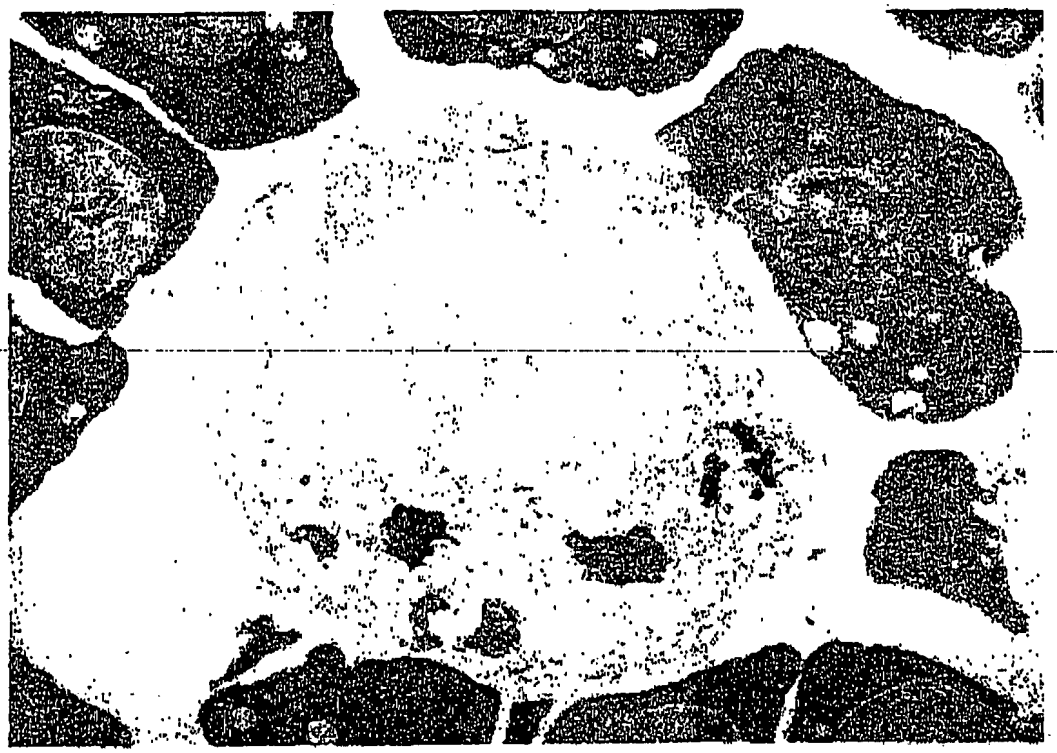

Fig. 11. A mitotic figure of a macrophage in the center of an erythroblastic island at 18 days of gestation. Peroxidase activity is observed in the rough endoplasmic reticulum, indicating that this is a resident macrophage. ×7,000.

tained from 10-day gestational-aged animals when grown on ST2 monolayer (Naito et al., 1990a,b). Macrophages in the early stage of colony formation show ultrastructural features similar to those of primitive macrophages and are positive for F4/80 and negative for peroxidase reaction. GM colonies are formed after the development of primitive or fetal macrophage colonies. In cell suspensions of fetal livers after 11 days of gestation, both primitive or fetal macrophage colonies (Fig. 17) and GM colonies (Fig. 18) develop, and the latter predominate over the former. Monocytes and granulocytes are most prominent in cell suspension cultures of fetal livers at 17 days of gestation. These results imply that macrophage progenitors in fetal hematopoiesis are different from those in adult animals, as are progenitors of primitive or fetal macrophages and those from the monocytic cell series in the early stages of fetal hematopoiesis. Primitive or fetal macrophage colonies are also formed in organ cultures of the yolk sac but not in semisolid or liquid cultures (Naito et al., 1990b). These facts may indicate that cell-to-cell contact is important for the development of primitive or fetal macrophages. For solving this problem, expression of CSF receptors and adhesion molecules on those progenitors seem to be of key importance.

KUPFFER CELL DIFFERENTIATION AFTER BIRTH

Based on the concept of MPS as proposed by Furth et al. (1972; Furth, 1989), all macrophages, including not only exudate macrophages discharged from inflammatory foci but also resident macrophages in tissues under a normal steady state, are considered to be derived from blood monocytes. Monocytes differentiate from promonocytes and monoblasts, which originate in the bone marrow (Furth, 1975, 1980, 1989, 1992; Furth et al., 1972, 1985). Monoblasts themselves are derived from M-CFCs originating from pluripotential myeloid hematopoietic stem cells. A monoblast divides once and gives rise to two promonocytes, and by a single division a promonocyte produces two monocytes. Thus, four monocytes are produced from a monoblast in the bone marrow. The production of monocytes in vivo is controlled by various growth factors, such as interleukin (IL)-6, IL-3, GM-CSF, and M-CSF. Among various CSFs, M-CSF is the most important for mediating the development and differentiation of a restricted macrophage lineage belonging to MPS. In other words, M-CSF-responsive macrophage populations are considered to belong to a member of the MPS.

In mice, monocytes leave the bone marrow within 24 hours after their production, enter the peripheral circu-

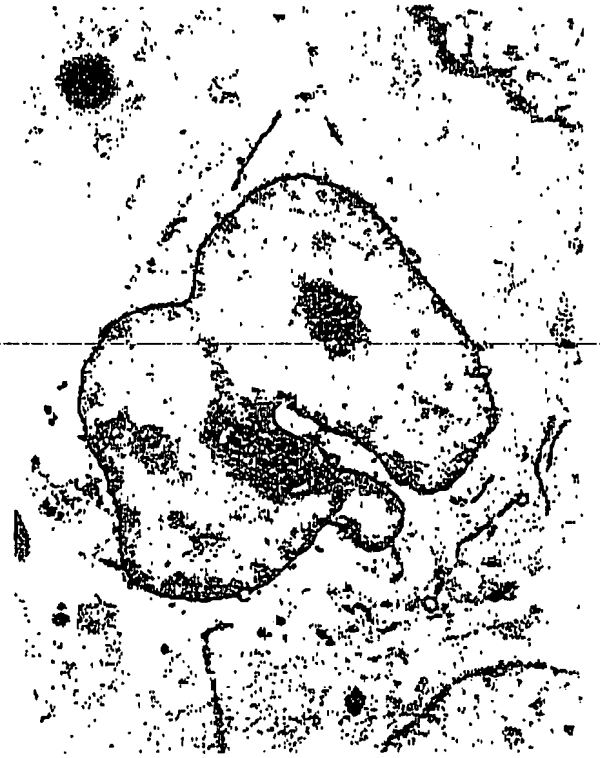

Fig. 12. Promonocyte in the fetal rat liver at 17 days of gestation. The cell has an indented nucleus and prominent nucleoli. Peroxidase activity is localized in the nuclear envelope, rough endoplasmic reticulum, and granules. ×8,000.

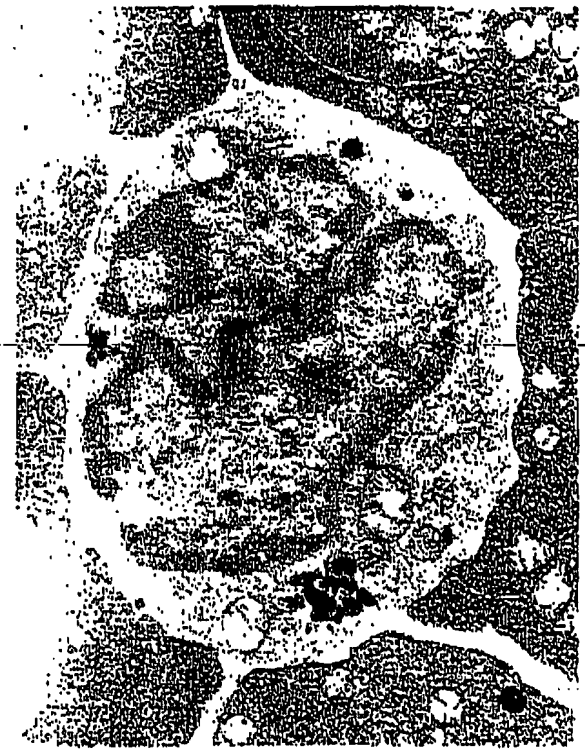

Fig. 13. Monocyte in the fetal rat liver at 18 days of gestation. The cell has a lobulated nucleus and peroxidase-positive granules. ×8,000.

lation, and circulate in the blood stream. The half-life of monocytes in circulation is 17.4 hours under normal conditions. They migrate into tissues and differentiate into macrophages in situ (Furth, 1975, 1980, 1989, 1992; Furth et al., 1972). Their influx rate is high in the liver and spleen but low in the lungs and peritoneal cavity (Furth, 1975; Furth et al., 1985). The life span of Kupffer cells is calculated to be 3.8 days. However, other data pertaining to Kupffer cell longevity conflict with these findings. After elimination of the cells by the administration of liposome-entrapped clodronate, Kupffer cells repopulate within 16 days in rats (Rooijen et al., 1990) and 14 days in mice (Yamamoto et al., 1990). In surviving rat liver grafts, donor Kupffer cells are completely replaced by recipient ones 15–30 days after grafting (Kaneda et al., 1991). In the mouse bone marrow transplant model, Kupffer cells of donor origin repopulate the recipient livers between 14 and 21 days (Paradis et al., 1989). In uncomplicated transplanted human livers, donor Kupffer cells persist up to 1 year (Steinhoff et al., 1989). Bouwens et al. (1986a,b) showed that the life span of resident macrophages in rats is from several weeks to 14 months. In ^{89}Sr-induced monocytopenic mice, Kupffer cells can be maintained for more than 6 weeks (Naito and Takahashi, 1991; Yamada et al., 1990). These data suggest that Kupffer cells are a long-lived resident macrophage population. However, the life span of Kupffer cells should be discussed in relation to their proliferative capacity and ultimate fate.

Little is known about the fate of Kupffer cells. Although monocyte production in the bone marrow is constant, Kupffer cell accumulation does not occur in the liver under physiological conditions. The number of monocytes in the peripheral blood generally exceeds the number required for replenishing dying macrophages in tissues (Ralph, 1989). If all of the monocytes enter the circulation and a large number of them reside in the hepatic sinusoid, Kupffer cells should die somewhere in the body or migrate to other sites before they die. A few days after the injection of colloidal carbon or gold into the portal vein for the selective labeling of Kupffer cells, labeled macrophages are found in the portal areas and in hepatic lymph nodes, suggesting that some Kupffer cells migrate to these sites (Hardonk et al., 1989).

Apoptosis (programmed cell death) has received much attention as one of the mechanisms that regulates the survival of monocytes and macrophages (Mangan et al., 1991, 1993a,b). In addition to several cytotoxic agents, apoptosis appears to be regulated by cytokines such as IL-1β, IL-4, and IFN (interferon)-γ. Tumor necrosis factor-α (TNF-α), GM-CSF, and IFN-γ prevent macrophage apoptosis in culture (Mangan et al., 1991, 1993a,b). IL-4 is known to induce apoptosis of monocytes in vitro (Mangan et al., 1993b). If it occurs in vivo,

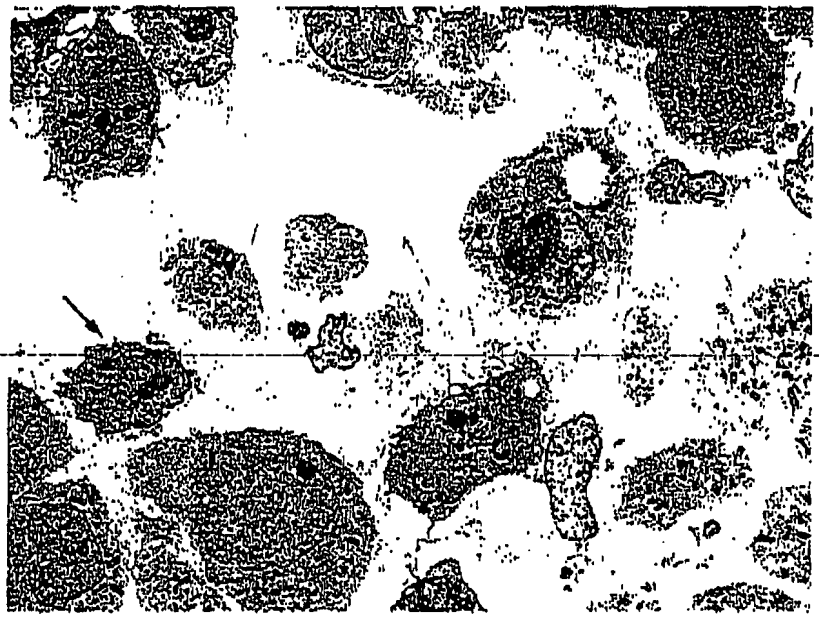

Fig. 14. Macrophages developing in the sinusoidal lumen of the human fetal liver. Arrow indicates a primitive macrophage. ×2,000.

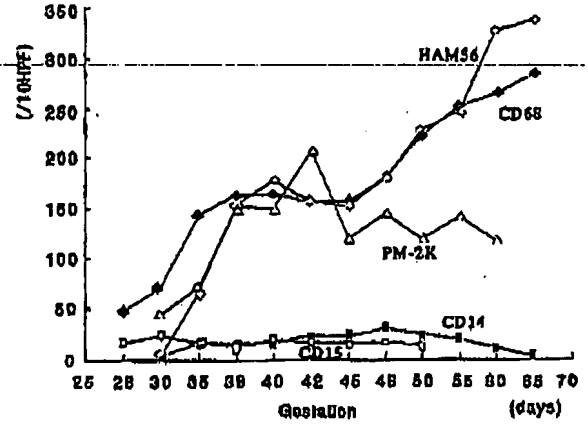

Fig. 15. Numbers of positive cells against various macrophage monoclonal antibodies per 10 high power field (HPF) in the human fetal liver.

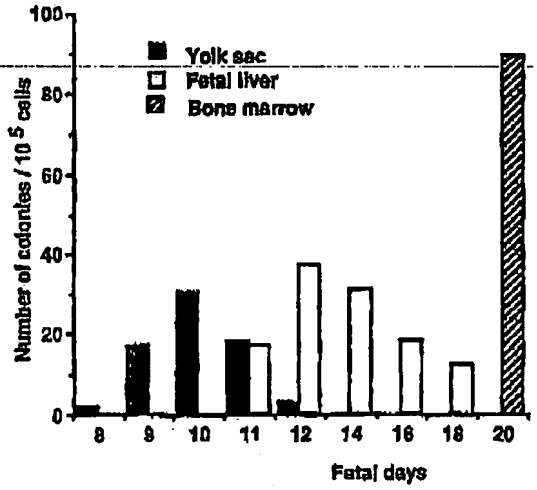

Fig. 16. Numbers of colony-forming cells in soft agar cultures of yolk sac cell suspensions with LP-3-conditioned medium from fetal days 8–11, liver cell suspensions from fetal days 11–18, and bone marrow cells from an adult mouse.

monocytes may die in circulation without their influx into tissues and differentiation into macrophages. In colony-forming assays, it has been shown that various CSFs promote macrophage survival by suppressing apoptosis (Williams et al., 1990). Because apoptotic cells are rapidly recognized by macrophages, Kupffer cells or monocytes undergoing apoptosis may be phagocytized and degraded by adjacent Kupffer cells. Such an efficient mechanism for controlling the cell death may occur to regulate the Kupffer cell population in a constant number within the liver.

KUPFFER CELL PROLIFERATION AFTER BIRTH

It has been widely accepted that monocyte-derived macrophages have no proliferative potential and die or disappear in tissues under a normal steady-state condition (Furth, 1975, 1980, 1989, 1992; Furth et al., 1972). In contrast, Kupffer cells in the adult liver and in fetal

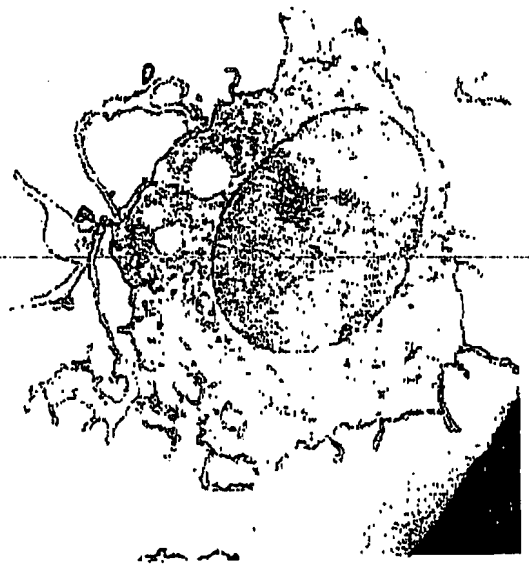

Fig. 17. Fetal macrophage development 5 days after culture of liver cell suspensions from a 10-day-old fetal mouse on a monolayer of ST2. The cell shows a positive reaction for F4/80 on the cell membrane and is negative for peroxidase reaction. ×5,000.

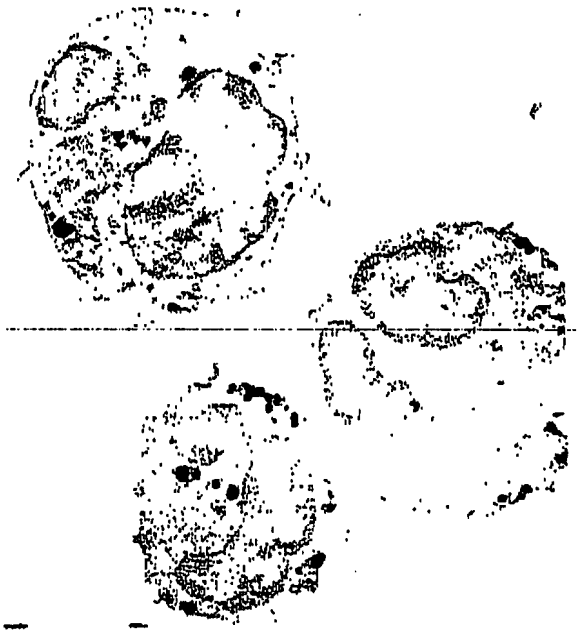

Fig. 18. Monocytes 5 days after culture of liver cell suspensions from a 10-day-old fetal mouse on a monolayer of ST2. The cells show a positive reaction for peroxidase in their granules. ×5,000.

livers have a proliferative capacity and are capable of surviving by self-renewal. By ^{3}H-thymidine autoradiography, the labeling rate of Kupffer cells is not high, about 2% in a normal steady-state condition. However, proliferation of Kupffer cells is enhanced in various experimental conditions. Because Kupffer cells show a low proliferative potential but a long lifespan, they might be maintained by self-renewal without a supply of monocytes from the peripheral blood.

To examine the significance of proliferation in Kupffer cell kinetics, we produced monocytopenic mice by the administration of strontium-89 (^{89}Sr). This method can induce severe monocytopenia in splenectomized mice without any damage to Kupffer cells for a long period of time (Naito et al., 1991; Yamada et al., 1990). In these mice, monocytes disappeared almost completely from the peripheral blood and the monocyte pool decreased to fewer than 1 in 200 (Yamada et al., 1990). However, the number of Kupffer cells in the monocytopenic mice was not reduced but rather was increased (Fig. 19) (Naito et al., 1991; Yamada et al., 1990). Because the number and proliferative capacity of Kupffer cells were increased in the monocytopenic mice for up to 6 weeks after ^{89}Sr administration (Naito et al., 1991), it appears likely that Kupffer cells can survive and be sustained by cell division without monocyte influx from the peripheral blood.

Proliferation of Kupffer cells by cell division is observed after the injection of various macrophage stimulators and after partial hepatectomy (Kojima, 1976; Widmann and Fahimi, 1975; Wisse, 1974b). Administration of glucan or zymosan induces both the influx of monocytes and Kupffer cell division (Delmann and Fahimi, 1980a,b; Bouwens et al., 1986a,b; Naito and Takahashi, 1991). These results also support the view that Kupffer cells are an independent, self-sustaining, and slow replicating cell population distinct from the MPS.

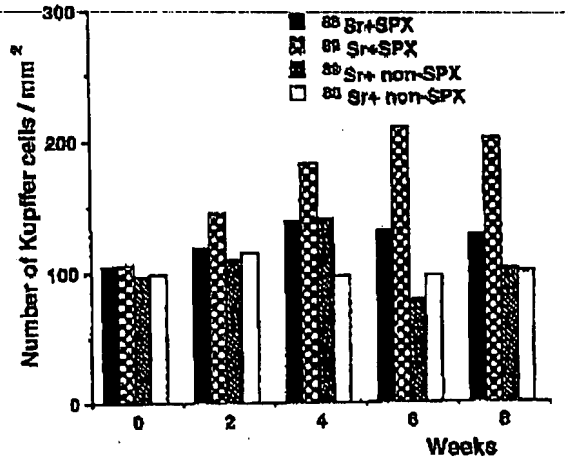

Fig. 19. Number of Kupffer cells in splenectomized (SPX) and nonsplenectomized (non-SPX) mice administrated strontium-88 or strontium-89.

REGULATION OF KUPFFER CELL DIFFERENTIATION AND PROLIFERATION BY CSFs

CSFs are essential molecules for the development and differentiation of hematopoietic cells. Immunophenotypical and ultrastructural studies have revealed that heterogeneous macrophages are produced in the colony-forming assays (Morioka et al., 1994; Wijffels et al., 1993). In the colony-forming assay, the size of M-CSF-derived macrophages is larger. They are more markedly differentiated, develop more abundant intracellular organelles, and have more extensive cytoplasmic projections than GM-CSF-derived or IL-3-derived macrophages. Such CSF-derived macrophages also show a marked difference in several functional properties (Rutherford et al., 1993). M-CSF, among other CSFs, is known to have a variety of effects on monocyte and macrophage lineage, such as increased production of monocytes and their precursors in hematopoietic organs, chemotactic activity for monocytes and macrophages, differentiation of monocytes into macrophage, induction of macrophage proliferation, and activation of endocytic and secretory functions. Mouse Kupffer cells have been shown to proliferate in vitro in the presence of glucan (Williams et al., 1989) or L929-conditioned medium (Chen et al., 1979). Glucan is known to induce the production of M-CSF and GM-CSF in Kupffer cells in vitro (Williams et al., 1989). Studies by Hoedemakers et al. (1994) have also demonstrated that although IL-2, IL-3, and IL-6 alone do not induce the proliferation of Kupffer cells, M-CSF and GM-CSF play important roles for the proliferation of Kupffer cells, especially large mature ones.

To examine the role of M-CSF on Kupffer cell development, differentiation, and proliferation in vivo, we used M-CSF-deficient animals. Osteopetrotic (*op/op*) mice are an animal model for osteopetrosis and develop peculiar osteosclerosis due to the complete absence of osteoclasts that are involved in resorption and remodeling of bones (Marks and Lane, 1976; Marks, 1982). Recently, it has been found that *op/op* mice are defective in the production of functional M-CSF proteins, resulting from a point mutation (thymidine insertion) that induces a defect in the coding region of the M-CSF gene (Yoshida et al., 1990). The mutant mice impair the differentiation of monocytes into macrophages (Naito et al., 1991; Yoshida et al., 1990) and have severe deficiencies of blood monocytes, Kupffer cells, and other tissue macrophages (Fig. 20). In various tissues of the mutant mice, the monocyte-derived macrophage population is completely depleted (Felix et al., 1990b; Wiktor-Jedrzejczak et al., 1982). However, there are varying numbers of M-CSF-independent macrophages in different tissues. The cells are small and round and reveal no cytochemical localization of peroxidase activity in any intracellular organelles. They are ultrastructurally immature (Fig. 21) and are characterized by having a small cytoplasm, poorly developed intracellular organelles, and a few short cytoplasmic projections (Naito et al., 1991; Usuda et al., 1994; Takahashi et al., 1994). In the mutant mice, the number of Kupffer cells is about 30% in normal littermates and they show active phagocytic function. Because GM-CSF levels in the mutant mouse are within the normal range (Wiktor-Jedrzejczak et al., 1990), the development and differentiation of such M-CSF-independent immature macrophages are regulated mostly by the effects of GM-CSF and thus are termed the "GM-CSF-dependent macrophage population" (Naito et al., 1991; Wiktor-Jedrzejczak et al., 1992). These results seem to provide evidence for the existence of a pathway of macrophage differentiation from GM colony-forming units or earlier macrophage

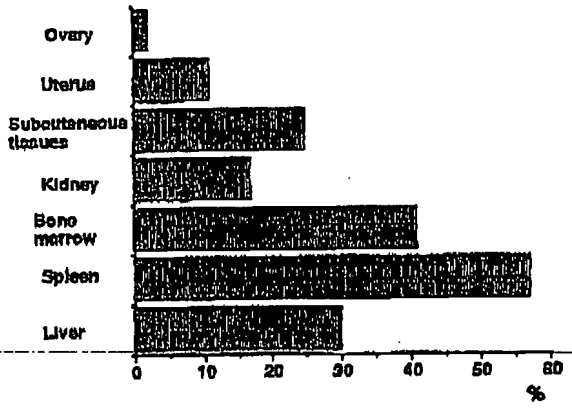

Fig. 20. Percentage of F4/80-positive macrophages in various tissues of *op/op* mice versus those of their normal littermates.

Fig. 21. Kupffer cell in an *op/op* mouse. The cell has only a few cytoplasmic projections, phagosomes, and vacuoles. Peroxidase activity is absent in the cell. ×6,000.

Fig. 22. Kupffer cell in an *op/op* mouse after the daily administration of M-CSF for 2 weeks. The cell shows well-developed ultrastructural features, and peroxidase reactivity is localized in the nuclear envelope and rough endoplasmic reticulum. ×6,000.

precursors, thereby bypassing the stage of monocytic cell series. It is of particular interest that the morphological features of GM-CSF-dependent macrophages in the mutant mice closely resemble those of primitive/fetal macrophages during ontogeny.

The administration of M-CSF into *op/op* mice drastically improves or cures osteosclerosis by the development, differentiation, and proliferation of osteoclasts (Felix et al., 1990a; Kodama et al., 1991, 1993; Takahashi et al., 1994; Wiktor-Jedrzejczak et al., 1991). The number of tissue macrophages and osteoclasts rapidly increases before the influx of monocytes, and their ultrastructure starts showing marked maturation toward resident macrophages. In this period, Kupffer cells also start developing peroxidase activity in the nuclear envelope and rough endoplasmic reticulum as seen in resident macrophages (Fig. 22). These findings indicate that, although there are 30% of GM-CSF-dependent Kupffer cells and 70% of M-CSF-dependent Kupffer cells in the liver, M-CSF acts not only on the lineage-specific differentiation of M-CSF-dependent monocyte and macrophage population but also on the maturation and proliferation of the GM-CSF-dependent Kupffer cell population.

We have studied the significance of M-CSF in protein-calorie malnutrition (Honda et al., 1995). In mice fed on a low-protein diet containing 4% casein, we found that the number of Kupffer cells decreased to two-thirds that of mice given a normal diet of equivalent calories containing 20% casein. They showed cytological and ultrastructural features of immature Kupffer cells similar to those in *op/op* mice and markedly reduced their proliferative capacity. After normal protein feeding or M-CSF administration, the number, morphology, and proliferative capacity of Kupffer cells returned to normal, and they matured as in normal-diet-fed mice. In the mice fed protein-restricted diet, the serum levels of IL-6 and GM-CSF increased, whereas messenger RNA of M-CSF was markedly reduced by Northern blot analysis. All these data indicate that M-CSF is a primary important molecule for the differentiation, maturation, and proliferation of Kupffer cells.

Recently, GM-CSF and M-CSF has been shown to be produced by not only sinusoidal cells, including Kupffer cells, but also hepatocytes in vitro (Sakamoto et al., 1990/1991; Tsukui et al., 1992). Besides soluble forms of M-CSF, M-CSF molecules associated with the cell membrane and a proteoglycan form have been reported, suggesting the significance of cell-to-cell or cell-to-intercellular matrix adhesion for macrophage differentiation and proliferation (Borojevic et al., 1993; Price et al., 1992; Suzu et al., 1992). Furthermore, the existence of hematopoietic stem cells in the adult liver has been suggested (Hays et al., 1978; Yamamoto et al., 1996). Besides the fetal period, extramedullary hematopoiesis is temporarily induced by various stimuli in the adult murine liver (Deimann and Fahimi, 1980a,b; Borojevic et al., 1993). We have observed that production of M-CSF in the liver is enhanced during Kupffer cell depletion, suggesting that locally produced M-CSF may induce proliferation and differentiation of repopulating Kupffer cells and their precursors (Yamamoto et al., 1996). These facts suggest that the adult liver is also capable of bearing hematopoietic activity and that CSFs play a significant role for providing a microenvironment for the differentiation and proliferation of Kupffer cells in not only the fetal liver but also the adult liver. The mechanisms of how CSFs are controlling the development, differentiation, maturation, proliferation of Kupffer cells and their function are an intriguing subjects that should be solved in further investigations.

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